The m6A Demethylase FTO Promotes Esophageal Cancer Progression Through YTHDF1-Dependent Posttranscriptional Silencing of AKT3

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Research

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Abstract

Background

N\textsuperscript{6}-methyladenosine (m\textsuperscript{6}A) is the most abundant modification in eukaryotic messenger RNAs (mRNAs), and plays important roles in many bioprocesses. However, its functions in esophageal cancer (ES) remain elusive.

Methods

Methylated RNA immunoprecipitation sequencing (MeRIP-seq) and transcriptomic RNA sequencing (RNA-seq) were used to screen the target genes of FTO. Western blot, quantitative real-time PCR (RT-qPCR) and immunohistochemical (IHC) were used to detect FTO expression in cell lines and patient tissues. The biological functions of FTO were investigated \textit{in vitro} and \textit{in vivo}. RNA pull-down and RNA immunoprecipitation assays were conducted to explore the specific binding of target genes.

Results

We discovered that the RNA demethylase FTO was significantly up-regulated in human ES. Knockdown of FTO drastically reduced ESCCs cell proliferation, migration, invasion, and apoptosis in the ESCCs. On the other hand, overexpression of FTO significantly promoted ESCCs growth and invasion. Moreover, we found that the m\textsuperscript{6}A methyltransferase METTL14 negatively correlates with FTO function on ES progression. By using transcriptome-wide m\textsuperscript{6}A-Seq and RNA-Seq assays, we identified AKT3 is the target of FTO, which acts in concert in esophageal cancer tumorigenesis and metastasis. Moreover, loss and gain functional studies confirm that YTHDF1 mediates m\textsuperscript{6}A-increased translation of AKT3 mRNA.

Conclusion

Our results uncovered an METTL14/FTO/YTHDF1/AKT3 signaling network that regulates the ES progression.

Background

The mRNA m\textsuperscript{6}A modification which has been identified in 1970s [1, 2], is the most abundant in eukaryotic mRNAs with unique distribution patterns [3]. In mammalian cells, The m\textsuperscript{6}A modification is catalyzed by a methyltransferase complex ("writers") consisting of the proteins methyltransferase-like 3 (METTL3), METTL14, Wilms tumor 1 associated protein (WTAP), VIRMA (KIAA1429), and RBM15 [4, 5]. Notably, the first RNA demethylase, fat mass and obesity-associated protein (FTO), was identified to function as an m\textsuperscript{6}A “eraser” to remove m\textsuperscript{6}A modification from RNA, revealing that RNA modification is reversible [6].
Afterwards, the alkylation repair homolog protein 5 (ALKBH5) was proved to be another “eraser” of m\textsuperscript{6}A modification [7], indicating a dynamic nature of m\textsuperscript{6}A methylation. Since then, numerous studies have been focused on the dynamics of m\textsuperscript{6}A modification [8–12]. Moreover, the m\textsuperscript{6}A-binding proteins with YTH domain, including cytoplasmic protein YTHDF1, YTHDF2, YTHDF3, and nuclear protein YTHDC1, have been identified to be the “readers” of m\textsuperscript{6}A and modulate mRNA stability and translation [9, 10, 13, 14].

Recently, the accumulated studies have been focused on the biological functions of m\textsuperscript{6}A modification in mRNA [15]. It has been reported that m\textsuperscript{6}A modification is involved in various biological processes, including heat-shock response[13], DNA damage response [16, 17], mRNA clearance [8], neuronal functions [18], cortical neurogenesis [19], progenitor cell specification [20], and T-cell homeostasis [21]. Moreover, m\textsuperscript{6}A modification has been found to be associated with the tumorigenesis and progression of various cancers [22].

The m\textsuperscript{6}A demethylase FTO was found to play critical roles in regulating fat mass, adipogenesis, and body weight [23–25]. In addition, large-scale epidemiology studies demonstrated the association of FTO SNP risk genotype with the development of cancers such as breast, kidney, prostate, and pancreatic cancers, as well as leukemia, lymphoma and myeloma [26–28]. The previous study showed that FTO plays an oncogenic role in cell transformation and leukemogenesis [29]. However, the definitive role of FTO in cancer remained limited.

In this study, we systematically investigated the role of m\textsuperscript{6}A modification in the tumorigenesis of esophageal cancer, which remains one of the most common form of cancer worldwide [30, 31]. We found a significant increased level of FTO in esophageal cancer cells. The following functional studies revealed an oncogenic role of FTO on ES tumorigenesis. Moreover, we found that m\textsuperscript{6}A methyltransferase METTL14 is also involved in the FTO-regulated m\textsuperscript{6}A modification, which acts in concert with FTO to regulate AKT3 methylation in the tumorigenesis and metastasis of esophageal cancer. Our findings provide hints that FTO/METTL14/AKT3 axis might be an effective therapeutic strategy to treat esophageal cancer.

**Materials And Methods**

**Samples, cell lines, and plasmids**

The human ESCCs samples obtained from abdominal surgery, Anhui Provincial Cancer Hospital, West Branch of the First Affiliated Hospital of USTC, and with patients’ informed consent. The pathological condition was determined by experienced surgical specialist. The comprehensive clinical and pathological information of ESCCs patients were shown in SI Appendix, Table S1. ESCCs cell line KYSE140, KYSE180, KYSE450, KYSE30, KYSE150 and human normal esophageal epithelial cell line HEEC were obtained from the Chinese Academy of Cell Resource Center (Shanghai, China) and maintained as previously described [32]. Cells were cultured in DMEM or RPMI 1640 (BI) medium supplemented with
10% fetal bovine serum (PAN), 100 U/ml penicillin, and 100 mg/ml streptomycin (Wisent) in humidified air at 37°C with 5% CO₂. All cell lines tested negative for mycoplasma contamination. FTO, METTL14, and AKT3 expressing lentivirus vector were purchased from Hanbio (Shanghai). Plasmids for expression of Flag tagged wild-type (YTHDF1-WT, YTHDF2-WT, YTHDF3-WT, YTHDC1-WT, YTHDC2-WT) were constructed with p3xFLAG-Myc-CMV vector. The detailed information regarding the primers used for plasmid constructs is depicted in SI Appendix, Table S2. For shRNA plasmids used in lentivirus-mediated interference, complementary sense and antisense oligonucleotides encoding shRNAs targeting FTO and were synthesized, annealed and cloned into pHBLV-U6-MCS-EF1-mcherry-T2A-PURO vector. The related sequences of shRNAs were shown in Table S2 of the SI Appendix.

**Gene expression and survival analysis in ESCCs cancer datasets**

Kaplan–Meier plotter (http://kmplot.com/analysis/) was used to assess the prognostic value of FTO and METTL14 expression in patients with ESCCs cancers. mRNA expression of FTO, YTHDC1, and YTHDF1 in cancer tissues and matched adjacent normal tissues of ESCCs cancers were obtained from TCGA (The Cancer Genome Atlas) database. GEPIA2 (http://gepia.cancer-pku.cn/) was used to assess correlation analysis of FTO and AKT3.

**m⁶A content analysis**

The content of m⁶A in total RNA was analyzed with the EpiQuik TM m⁶A RNA Methylation Quantification Kit (Epigentek).

**Dot-blot assays**

The mRNA was obtained according to the PolyATtractR mRNA Isolation System kit (Promega) instruction manual. 50ng/100ng mRNA was diluted to 2 µl with DEPC water and placed in a PCR instrument for thermal denaturation at 65°C for 10 minutes. The denatured RNA was evenly dotted onto the positively charged nylon membrane (Beyotime). UV irradiation was placed under the operating platform for 15 minutes, and RNA was fixed to the membrane. The fixed nylon film was washed in 1 x PBST for 3 times, 5 minutes each time. 10 ml of 5% skim milk sealant was prepared, and the membrane was placed in the sealant and sealed at room temperature for 2 hours. Rabbit m⁶A primary antibody (Active motif) was formulated at ratio of 1 :1000. Dip the nylon membrane in the primary antibody and incubate overnight at 4°C. The nylon film incubated overnight was washed with PBST for 3 times, 5 minutes each time. Rat anti - rabbit secondary antibody was prepared according to 1:2000. The nylon membrane was immersed in the secondary antibody and incubated at room temperature for 2 hours. The nylon film was washed 4 times with TBST, 5 minutes each time. The ECL color solution was prepared, and the nylon film was immersed in the color solution for 10 seconds. The nylon film was taken out and observed under the developer. Then the nylon film was stained with 0.2% methylene blue dye (pH5.2, corrected by 0.3 M sodium acetale for pH) for 0.5 hours. Photograph was taken and the sample load volume of each sample was compared.
m\textsuperscript{6}A-RT-PCR

m\textsuperscript{6}A-RT-PCR was conducted according to previously described protocol with a slight modification [33]. Briefly, the total RNA was extracted using Trizol and fragmented by RNA fragmentation reagents (Thermo, AM8740) or not. After saving 50 ng of the total RNA as input, the remaining RNAs (2 µg) were used for m\textsuperscript{6}A-immunoprecipitation with m\textsuperscript{6}A antibody (Synaptic Systems) in 500 µl of IP buffer (150 mM NaCl, 0.1 % NP-40, 10 mM Tris, pH 7.4, 100 U RNase inhibitor) to obtain m\textsuperscript{6}A pull down portion (m\textsuperscript{6}A IP portion). m\textsuperscript{6}A RNAs were immuno-precipitated with Dynabeads ® Protein A (ThermoFisher Scientific) and eluted twice with elution buffer (5 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.05% SDS, 20 mg/ml Proteinase K). m\textsuperscript{6}A IP RNAs were recovered by ethanol precipitation, and RNA concentration was measured with Qubit® RNA HS Assay Kit (ThermoFisher Scientific). Then 2 ng of the total RNA and m\textsuperscript{6}A IP RNA were used as templates in qRT-PCR, as described above.

In vitro cell proliferation, migration, and invasion assays

Cell proliferation was measured using the CCK-8 according to the manufacturer's instructions, 5 × 10\textsuperscript{3} cells per well were seeded onto a 96-well plate and checked every 24 hours (0, 24, 48, 72 and 96 hours). Triplicated samples were counted.

The cell migration test was performed in a 24-well plate with 8 mm pore size transwell chamber (Corning). 200 µl of a suspension containing 5 ×10\textsuperscript{4} cells made of RPIM 1640 without FBS was inoculated into the upper part of the chamber. 600 µl of RPIM 1640 medium containing 20% FBS was added to the lower part of the chamber. After incubating for 30 hours at 37°C and 5% CO\textsubscript{2}, take out the transwell chamber, discard the culture medium in the well, wash twice with PBS, fix with methanol for 5 minutes, stain with 0.1% crystal violet for 30 minutes, wipe off the upper layer of cells with a cotton swab, wash with PBS 3 times, randomly take 5 fields of view under the microscope to observe the cells and count them.

Cell invasion assays were performed in a 24-well plate with 8 mm pore size chamber inserts (Corning). 8 ×10\textsuperscript{4} cells were seeded in the upper portion of the invasion chamber with 200 µl of RPIM1640 without FBS. The lower portion of the chamber contained 600 µl of medium supplemented with 20 % FBS and glutamine. After incubation for 36 hours at 37°C and 5% CO\textsubscript{2}, the non-invading cells were removed from the upper surface of the membrane. Cells that moved to the bottom surface of the chamber were stained with 0.1% crystal violet for 30 minutes. The cells were then imaged and counted in four separate areas with an inverted microscope.

RNA extraction and real-time PCR for gene expression
RNA extraction with Trizol (Invitrogen) and real-time PCR were performed according to the protocol used in our previous study [34]. Primers of targeted genes were depicted in SI Appendix, Table S2.

**Colony formation assays**

For the colony formation assay, about 500 infected or transfected cells were seeded into each well of a 6-well plate and maintained in a medium containing 10% FBS for 10 days. The colonies were fixed with methanol and stained with 0.1% crystal violet, and the number of clones was counted. Triplicated samples were counted.

**Cell apoptosis assays**

Cells were harvested and rinsed twice with pre-cooling PBS. The samples were diluted with 150 µl of 1×annexin-binding buffer, then 5 µl of APC-labeled enhanced annexinV and 5 µl (20 µg/ml) of propidium iodide (PI) were added. Then the cells were incubated in the dark for 15 minutes at room temperature. Flow cytometry was conducted on a FACSCalibur instrument (BD).

**Wound-healing assay**

For wound-healing assay, cells were seeded and cultured until a 90% confluent monolayer was formed. Cells were then scratched by a sterile pipette tip and treated as indicated in the text in the FBS-free medium. Cell migration distances into the scratched area were measured in 10 randomly chosen fields under a microscope.

**Western blot assays**

Cells were harvested and washed twice with PBS. After adding lysis buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.25% bromophenol blue and 1.25% 2-mercaptoethanol), samples were lysed and heated for 10 minutes at 95°C, followed by centrifugation at 4°C and 13,000 rpm for 30 minutes. Whole-cell proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene fluoride (PVDF). Membranes were blocked with 5% nonfat milk at room temperature for 1 hour or at 4°C overnight and then incubated with the appropriate antibody. Images were captured by the Image Reader. Detailed information regarding the full-length gels is depicted in Supplementary Figures of SI Appendix.

**Biotin RPD assays**

RPD assays were performed using the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher) according to the manufacturer’s instructions. KYSE150 cells were transfected with biotinylated AKT3 sense probe and antisense probe (50 µl streptavidin beads were washed once with RPD buffer, then 10 µl of sense probe and 10 µl of antisense probe were added, and incubated for overnight at 4°C). and the cells were incubated at 4°C for 1–3 hours and the total cell lysates were incubated at room temperature for 2 hours. The bead-RNA-protein complexes were washed with 1 × binding-washing buffer four times. The proteins were precipitated and diluted in protein lysis buffer. Finally, the retrieved proteins were
measured by real-time PCR and/or western blot analysis. Detailed information regarding the primers used for real-time PCR analysis is depicted in SI Appendix, Table S2.

**RNA immunoprecipitation (RIP) assays**

RIP was performed with Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the instructions provided by the manufacturer [35]. Briefly, approximately $2 \times 10^7$ KYSE150 cells were lysed in hypotonic buffer supplemented with RNase inhibitor and protease inhibitor before centrifugation. The lysates were incubated with magnetic beads and coated with the indicated antibodies for 4 hours or overnight at 4°C. Then, the RNA-protein complexes were washed 6 times and incubated with proteinase K digestion buffer, the bead-bound immunocomplexes were treated with proteinase K for 30 minutes at 55°C. Samples were centrifuged and placed on a magnetic separator, and supernatants were used to extract RNA by Kit (Bioline). Purified RNAs were then subjected to PCR analysis and normalized to the input. Detailed information regarding the primers used for PCR analysis is depicted in SI Appendix, Table S2.

**m$^6$A sequencing (m$^6$A-seq) and data analysis**

The total polyadenylated RNA was isolated from KYSE150 and KYSE30 FTO knockdown (sh-FTO) cells using Trizol reagent (Tiangen). RNA fragmentation, m$^6$A-seq, and library preparation were performed according to the manufacturer’s instructions [36]. RNA Library Prep Kit (NEB, USA) was used for library preparation. Each experiment was conducted with two biological replicates. m$^6$A-seq data were analyzed according to protocols. Significant peaks with FDR < 0.05 were annotated to RefSeq database (hg19). Sequence motifs were identified by using Homer. Gene expression was calculated by Cufflinks using sequencing reads from input samples. Cuffdiff was used to find DE genes.

**Vector and m$^6$A mutation assays**

The potential m$^6$A sites were predicted using an online tool, SRAMP (http://www.cuilab.cn/sramp/). Full-length AKT3 transcripts, the AKT3 CDS region, the AKT3 three prime untranslated region (3'UTR), and the m$^6$A motif depleted CDS or 3'UTR regions were cloned into pcDNA3.1 for the RNA pull down assay. The specific sequences are shown in SI Appendix, Table S2.

**RNA stability**

To measure RNA stability of FTO knockdown to KYSE150 cells, actinomycetes D (6 µg/ml) treated control cells and down-regulated FTO cells to block RNA transcription at 0, 2, 4, 6, 8 hours, respectively. AKT3 mRNA residue was detected by quantitative PCR and the stability of mRNA was calculated.

**Luciferase reporter assays**

Luciferase assay was performed using reporter lysis buffer (Promega) and luciferase assay reagent according to the manufacturer’s instructions. Briefly, FTO knockdown-KYSE150 cells were transfected with pGL3, pGL3-WT-3’UTR, pGL3-Mut1-3’UTR, or
pGL3-Mut2-3’UTR in a 6-well plate. After transfection for 8 hours, each cell line was re-seeded into a 96-well plate. After 24 hours incubation, both firefly and Renilla luciferase activities were measured 24 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega) and a Promega GloMax 20/20 luminometer. The relative firefly luciferase activities of the UTR construct and pathway reporter constructs were analyzed as previously reported [37]. Detailed information regarding the primers used for plasmid construction is depicted in SI Appendix, Table S2.

**In vivo xenografts model**

Four-week-old male BALB/c nude mice were purchased from Zhejiang Weitong Lihua Laboratory Animal Technology Co., Ltd. The nude mice were kept in the SPF Animal Laboratory of China University of Science and Technology. Animal experiments were conducted in accordance with the National Guidelines for the Health Use of Laboratory Animals. Animal research was approved by the Biomedical Ethics Committee of China University of Science and Technology. The procedures for mouse experiments were implemented in accordance with the Regulations on the Administration of Laboratory Animals approved by the State Council.

For subcutaneous transplanted model, sh-control, sh-FTO, NC-OE and AKT3-OE KYSE150 cells (6 × 10^6 per mouse, n = 3 for each group) were diluted in 100 µl of PBS + 100 µl Matrigel (BD) and subcutaneously injected into immunodeficient male mice to investigate tumor growth. When tumor volume, in each group, reached ~ 100 mm^3, all the mice were killed, and tumors were removed and weighed for use in immunohistochemistry assays and further studies. The tumor volume was calculated using the equation V = 0.5×D×d² (V: volume, D: longitudinal diameter, d: latitudinal diameter). For in vivo lung metastasis model, mice were injected with WT (wide-type), sh-FTO, AKT3-OE and sh-FTO + AKT3-OE KYSE150 cells (1 × 10^6 per mouse, n = 3 for each group). six weeks after injection, mice were killed and metastatic lung tumors were analyzed.

**Immunohistochemistry assays**

The protein expression levels of Vimentin, E-cadherin, and MMP2 were determined by immunohistochemistry (IHC). IHC staining was performed on 4-mm sections of paraffin-embedded tissue samples. Briefly, the slides were incubated with the appropriate antibody at 4°C overnight. The subsequent steps were performed using the GTVision III Detection System/Mo&Rb (GeneTech). Detailed information regarding the antibody is depicted in SI Appendix, Table S2.

**Statistical analysis**
Statistical analysis was carried out using Microsoft Excel software and GraphPad Prism to assess differences between experimental groups. Statistical significance was analyzed by a two-tailed Student’s t test and one-way ANOVA. \( p \) values less than 0.05 were considered to be statistically significant: *, \( p \) value < 0.05; **, \( p \) value < 0.01; ***, \( p \) value < 0.001.

Results

The FTO expression is upregulated in esophageal cancer cells

We first investigated the \( m^6A \) levels in mRNAs of ESCCs. By using MeRIP-PCR, we identified that the \( m^6A \) levels of mRNAs isolated from five ESCCs, were statistically (\( p < 0.05, t \) test) less abundant than that of one normal control cell line (Fig. 1A). The \( m^6A/A \) level of mRNA are decreased to ~ 10% in ESCCs, compared to ~ 40% of the normal cells (Fig. 1A). The results indicates that the \( m^6A \) in mRNAs should be demethylated in the ESCCs. We thus detected the expression of the N\(^6\)-methyladenosine RNA demethylase FTO in the tissues of different ES patients. Compared to the normal tissues, the tissues from the esophageal cancer patients had an up-regulated level of FTO expression (Fig. 1B). Moreover, the ES patients with higher FTO expression had a shorter overall survival (Fig. 1C), which suggests that FTO expression might serve as a prognostic marker for ES patients. The following western blotting analysis also showed a significantly higher expression of FTO in ESCCs, especially in KYSE150 cells, compared to the normal ES cells (Fig. 1D). In addition, the ES patients had a higher expression of FTO proteins, as analyzed by western blotting (Fig. 1E). The expression of FTO is also significantly higher in the ES tissues, compared to the para cancer tissues (PT) (Fig. 1F). Collectively, all these results clearly demonstrated that FTO is highly expressed in the ES patients, which correlates with a lower survival rate of ES patients.

FTO is involved in the cell proliferation, migration, invasion, and apoptosis in the ESCCs

To investigate the potential roles of FTO in ESCCs, we performed a series of functional assays to characterize the effect of FTO in ESCCs. We first down-regulated the expression of FTO by transfection of FTO sh-RNAs in KYSE150 cells (Fig. 2A). The following CCK-8 assays showed that down-regulation of FTO significantly inhibited the cell proliferation of KYSE150 cells (Fig. 2B). Moreover, the FTO knockdown in KYSE150 cells decreased the cell migration and invasion capability compared to that of the control cells (Fig. 2C). The previously studies showed that E-cadherin is a biomarker for cell migration [38, 39], we thus detected the expression of E-cadherin in FTO knockdown cells. The results showed that down-regulation of FTO correlates with the higher expression of E-cadherin (E-cad) in KYSE150 cells (Fig. 2D), indicating that FTO might associate with the E-cadherin-regulated cell migration in ESCCs.

Afterwards, we overexpressed FTO in HEEC cells by transfection of FTO-overexpressing lentivirus (Fig. 2E). Resultantly, the cell proliferation rate was significantly increased upon overexpression of FTO in
HEEC cells (Fig. 2F). Moreover, the cell migration and cell invasion capability is also increased in FTO-overexpressed HEEC cells (Fig. 2G). In addition, the colony formation assays showed that compared to the control cells, FTO overexpression significantly promoted cell proliferation in KYSE150 cells, whereas FTO knockdown largely impedes the cell proliferation (Fig. 2H). Moreover, compared to the control cells, FTO overexpression in HEEC cells drastically decreased the apoptotic cells from 25–10%, as analyzed by flow cytometry (Fig. 2I), indicating a decreased cell apoptosis rate with FTO overexpression. All these results indicated that the oncogenic role of FTO involved in the cell proliferation, migration, invasion, and cell apoptosis in ESCCs.

**FTO negatively correlates with METTL14 in ESCCs**

Our results showed that a higher FTO level correlates with a poor prognosis in ES patients (Fig. 1C). To further characterize whether m$^6$A methylation is indeed associated with the ES prognosis, we introduced the ratio of FTO/METTL14 to represent the methylation rate in the cells. Given METTL14 is the well-characterized m$^6$A methyltransferase [40, 41], thus the higher FTO/METTL14 ratio represents the lower level of m$^6$A methylation in the cells. Analysis of ES patients with higher FTO/METTL14 ratios showed a rather poor prognosis rate, compared to that of lower FTO/METTL14 ratios (Fig. 3A). Further analysis of the differentially expressed genes by RNA-seq showed that FTO-overexpression and METTL14-overexpression have totally 157 shared differentially expressed genes (Fig. 3B). These results indicated that FTO might be functionally associated with METTL14. To further examine the correlation between FTO and METTL14, we compared the proliferation ability of KYSE150 cells with the reversal changes of FTO or METTL14. As expected, down-regulation of FTO or up-regulation of METTL14 reduced the proliferation ability, which could be restored via down-regulation of METTL14 (Fig. 3C). Moreover, the decreased migration or invasion of KYSE150 cells via FTO knockdown could also be restored by METTL14 overexpression (Fig. 3D). By contrast, METTL14 knockdown has an opposite effect of an elevated capability of migration or invasion in KYSE150 cells. All these results suggest that FTO and METTL14 negatively correlate with each other in functioning as biomarkers in ES patients.

**AKT3 is the target of FTO in ESCCs**

To further identify the potential targets of FTO in ESCCs, we detected the m$^6$A contents of the total mRNA analysis with EpiQuik™ m$^6$A RNA Methylation Quantification Kit (Colorimetric) in FTO-overexpressed HEEC and FTO-silenced KYSE150 cells (Figs. 4A-4B). As expected, FTO-overexpression significantly decreased the m$^6$A content in HEEC cells whereas FTO-silencing dramatically increased the m$^6$A content in KYSE150 cells (Figs. 4A-4B). As analyzed by RMBase database, the genes with m$^6$A modification has a consensus motif of U/AGGAC (Fig. 4C), which is the common feature among the genes with m$^6$A methylation [42, 43]. Compared to the control cells, a total of 128 genes, showing a 1.5-fold m$^6$A change in the expression level, were identified ESCCs cells (Fig. 4D). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of these 128 genes indicated that a handful of genes were associated to the different metabolic pathways in various cancer cells (Fig. 4D). The overall level analysis of the modification of m$^6$A after FTO knockout indicated that FTO was related to the methylation of m$^6$A.
Among these genes, we selected the top seven most differently expressed members and detected their expressions in KYSE150 cells by real-time PCR. The results showed that METTL1, CAMKK1, PKN2, and TGFBR1 were up-regulated in FTO-silenced KYSE150 cells, whereas ERBB2, AKT3 and PAK1P1 were down-regulated (Fig. 4F). Among the three down-regulated genes, AKT3 which is serine/threonine kinase from the AKT family [44, 45], is involved in the biogenesis of many different types of cancers [46].

We thus detected the m\(^6\)A abundances on AKT3 mRNA transcripts in KYSE30 and KYSE150 cells by m\(^6\)A-seq (Fig. 4G). The results showed that the m\(^6\)A methylation is enriched in the exon and 3′UTR regions of AKT3 with a clustered distribution (Fig. 4H). The correlation prediction of FTO and AKT3 by the GEPIA database gave as R-value of 0.55, which strongly indicated the physical interaction between FTO and AKT3 (Fig. 4I).

To further investigate the role of AKT3 in FTO-regulated m\(^6\)A methylation, we detected the m\(^6\)A content in KYSE150 cells by MeRIP-PCR. The results showed that FTO knockdown retained the m\(^6\)A methylation in AKT3, as shown by an elevated m\(^6\)A content in KYSE150 cells (Fig. 4H). To examine the role of m\(^6\)A methylation on AKT3 3′UTR region, we generated luciferase reporters containing a firefly luciferase, followed by the wild-type AKT3 3′UTR, mutant1 or mutant2 3′UTR. The 3′UTR-reporter luciferase assay showed that compared to the control cells, the mutant1 3′UTR slightly but not significantly reduced AKT3 expression, whereas the mutant2 3′UTR has a minor effect on AKT3 expression (Fig. 4J). Dot plot analysis of the overall level of m\(^6\)A modification in esophageal cancer cells before and after knockdown of AKT3 m\(^6\)A modification site. The results indicated that the m\(^6\)A methylation on 3′UTR might not be involved in the m\(^6\)A modification-regulated AKT3 expression.

**AKT3 is involved in m\(^6\)A-regulated esophageal cancer tumorigenesis and metastasis**

We then characterized the roles of AKT3 on ESCCs cell functions by several in vitro experiments. Notably, overexpression of FTO in HEEC cells largely increased the expression of both AKT3 mRNA and protein (Fig. 5A), whereas FTO knockdown in KYSE150 cells decreased the expression AKT3 mRNA and protein (Fig. 5B). To further investigate whether FTO affects the stability of AKT3 mRNA, we tested the AKT3 mRNA levels in KYSE150 cells with FTO knockdown after the treatment with Actinomycete D, which is a metabolic inhibitor [38, 47]. The results showed that the mRNA level is dramatically decreased along the time with FTO knockdown (Fig. 5C), indicating that FTO might increase the stability of AKT3 mRNA, which results in a higher level of AKT3 protein expression.

Next, we down-regulated the AKT3 expression in KYSE150 cells and tested its effect on KYSE150 cellular functions. Transfection of either of the three AKT3 siRNAs in KYSE150 cells significantly decreased the expression of AKT3 in both mRNA and protein levels (Fig. 5D). In addition, overexpression of AKT3 also increased the expression of Vimentin (Fig. 5E), indicating that AKT3 promotes tumor cell migration. Accompanied by the decrease of AKT3 with si-AKT3 transfection in KYSE150 cells, the cell proliferation ratio is also decreased along the time (Fig. 5F). Moreover, the cell invasion and migration capabilities are
also decreased with AKT3 knockdown in KYSE150 cells (Fig. 5G). All these results indicated that AKT3 involves in the tumorigenesis of ES progression.

**FTO and AKT3 act in concert to regulate esophageal cancer tumorigenesis and metastasis**

To further investigate the correlation between FTO and AKT3 on esophageal cancer tumorigenesis, we overexpressed AKT3 in KYSE150 cells accompanied with the knockdown of FTO. As shown in Fig. 6A, compared to the transfection of AKT3-overexpressed vector (AKT3-OE) that increased the AKT3 mRNA expression to ~43.5-folds, the AKT3 expression in FTO-silenced KYSE150 cells with transfection of AKT3-overexpressed vector (sh-FTO + AKT3-OE) increased to ~15.3 folds, which largely compromises the up-regulation of AKT3 mRNA. The results also coincide with the notion that FTO-regulated m\(^6\)A demethylation promotes the AKT3 mRNA stability (Fig. 5C). As a result, the AKT3 protein levels are also up-regulated in the AKT3-OE and sh-FTO + AKT3-OE KYSE150 cells (Fig. 6B), but a little bit lower in the sh-FTO + AKT3-OE cells. To test whether AKT3 could restore the effect of FTO knockdown, we tested the proliferation ratio of KYSE150 cells using CCK-8 assays. The results showed that FTO knockdown decreased the proliferation ratio, which could be restored by AKT3 overexpression in the sh-FTO + AKT3-OE cells (Fig. 6C). Similarly, the wound healing, migration, invasion and colony formation assays showed that the effects of FTO knockdown in KYSE150 cells could also be restored by AKT3 overexpression (Figs. 6D-6G). These results also indicated that FTO-regulated m\(^6\)A demethylation on AKT3 is associated with the tumorigenesis and metastasis of esophageal cancer cells.

**YTHDF1 maintains AKT3 mRNA stability via an m\(^6\)A-dependent manner**

Previous studies had identified two major families of m\(^6\)A “readers” that might play a specific role in control the fate of the methylated mRNA, such as the YTH family and the IGF2BP family[5, 48, 49]. To identify the specific m\(^6\)A readers of AKT3, and determine the m\(^6\)A-dependent mechanism of AKT3 regulation, we performed a FLAG RNA pull-down assays in KYSE150 cells to screen the AKT3-related m\(^6\)A readers. Notably, YTHDC1 and YTHDF1, but not other members in the YTH family, specifically bind to the AKT3 full-length transcripts in KYSE150 cells (Fig. 7A). The biotin-based pull-down assays also confirmed the direct interactions of AKT3 mRNA with both YTHDC1 and YTHDF1 (Figs. 7B-7C), indicating the potential positive regulatory mechanism. Further detection of the expression levels in the tumor tissues compared to the control groups revealed that only YTHDF1 had an up-regulated expression level in ESCCs (Fig. 7D). In contrast, YTHDC1 has no significant changes of the expression level compared to the control groups (Fig. 7E).

To further test the role of YTHDF1 on AKT3 stability, we inhibited or increased the expression of YTHDF1 in KYSE150 cells. Resultantly, the AKT3 mRNA and protein expression were significantly decreased upon the overexpression of YTHDF1 in KYSE150 cells (Fig. 7F). Moreover, the AKT3 mRNA and protein expression were up-regulated after the siRNA inhibition of YTHDF1 in KYSE150 cells (Fig. 7G).
together, our results suggested that the methylated AKT3 transcripts might directly recognized by YTHDF1, which maintained the stability of the AKT3 transcripts.

**FTO and AKT3 promote esophageal cancer progression *in vivo***

To test the potential role of FTO and AKT3 on esophageal cancer biogenesis *in vivo*, we injected the sh-FTO or AKT3-OE KYSE150 cells subcutaneously into the nude mice. Then the mice were killed when the tumor volumes were about 100 mm$^3$ for each group. Compared to the control groups, transfection of sh-FTO KYSE150 cells significantly decreased the tumor weight whereas transfection of AKT3-OE KYSE150 cells increased the tumor weight (Fig. 8A). The following IHC results showed that FTO depletion can decrease the EMT potential whereas AKT3 overexpression increased the EMT potential (Fig. 8B). To further determine the impacts of m$^6$A methylation on *in vivo* metastasis, sh-FTO, AKT3-OE or sh-FTO&AKT3-OE KYSE150 cells were injected into the nude mice, respectively, by tail vein injection to analyze lung colonization. As shown in Fig. 8C, the number of lung tumors derived from FTO knockdown KYSE150 cells showed no significant changes; however, AKT3-OE or sh-FTO&AKT3-OE significantly promoted the number of lung tumors compared with control cells, suggesting that AKT3 overexpression promoted tumor metastasis in vivo. Further analysis revealed that a lower level of VIM and MMP2 in xenograft tumor tissues with transfection of sh-FTO or sh-AKT3 KYSE150 cells, whereas their expression was up-regulated upon FTO or AKT3 overexpression. However, the expression of E-cad was opposite (Fig. 8D). Moreover, FTO and AKT3 could restore the function with each other, as shown by the comparable level of these proteins when transfection with either sh-FTO&AKT3-OE or FTO-OE&sh-AKT3 KYSE150 cells. All these results suggested that FTO and AKT3 are involved in the esophageal cancer progression *in vivo*.

**Discussion**

Increasing evidences indicated that m$^6$A mRNA modification participates in a number of biological functions and in progression of cancer cells [29, 38, 50]. In this study, we demonstrated that m$^6$A can regulate the progression of esophageal cancer. In brief, the decreased m$^6$A levels in mRNA of esophageal cancer is correlated with the higher level of FTO. A reversal changes of FTO and METTL14 levels largely affect the in vitro proliferation, migration, and invasion of cancer cells. Further investigations identified that AKT3 is one of the targets of FTO. Particularly, AKT3 is a phosphatidylinositol-3-kinase which protein kinase B family, is a key element of the PI3K/AKT signaling pathway. The AKT pathway was found to regulate many hallmarks of cancer and the metastatic cascade in breast cancer [51–53]. In addition, much effort was made to develop targeted therapy for AKT signaling in breast cancer [54–56]. Thus PI3K/AKT pathway is a promising target for cancer therapy owing to the high frequency of its dysregulation in human breast cancer [57]. Here we showed that AKT3 is involved in the progression of
esophageal cancer, which provide the basis for further targeting AKT3 pathway for clinical therapy of esophageal cancer.

The knowledge on the roles of mRNA modification in controlling the cancer progression remains limited. FTO, the first characterized m\(^6\)A demethylase, has been reported to regulate the tumorigenesis of different types of cancers. FTO was found to enhance leukemic oncogene-mediated cell transformation and leukemogenesis via reducing the m\(^6\)A levels of its targets [29]. In addition, pharmaceutical inhibition of FTO by a chemical inhibitor suppresses tumor progression and substantially prolongs the lifespan of glioblastoma stem cell-grafted mice [50]. On the other hand, METTL14, which is the methyltransferase of mRNA m\(^6\)A, exhibits several functions in cancer cells, such as: regulating leukemogenesis and proliferation of hematopoietic stem/progenitor cells (HSPCs) [41]. Targeting METTL14, especially in combination with differentiation-inducing agents, may represent effective novel therapeutic strategies to treat AMLs. In addition, METTL14, which forms a stable heterodimeric core complex with METTL3 to function in cellular m\(^6\)A deposition, can suppress metastatic potential by modulating the primary microRNA 126 processing in an m\(^6\)A-dependent manner [58]. In this study, we provided the link between the regulation of AKT3 mRNA m\(^6\)A methylation by FTO and METTL14. We found that down-regulation of FTO or overexpression of METTL14 have a similar effect on the multiple aspects of esophageal cancer progression, including migration, invasion, proliferation, and tumorigenesis, which also suggests that the FTO function could be restored by METTL14 in esophageal cancer. Our results described the roles of m\(^6\)A and FTO in cancer progression, and also provide the basis to develop therapeutic strategies against esophageal cancer metastasis by targeting m\(^6\)A modification and its related targets.

The m\(^6\)A modification modulates all stages in the life cycle of RNA, such as RNA processing, nuclear export, and translation modulation [14, 59, 60]. For example, m\(^6\)A modification can trigger mRNA degradation by promoting deadenylation of RNAs through the first characterized m\(^6\)A “reader” protein YTHDF2 [9]. Here we showed that the stability of AKT3 mRNA transcripts is enhanced by the reader protein YTHDF1. The previous studies showed that YTHDF1 links with the progression of various cancers, including non-small cell lung cancer [61], Colorectal Carcinoma [62], and ovarian cancer [63]. In our study, we found that knockdown of YTHDF1 decreased the AKT3 in both mRNA and protein levels (Fig. 7F), whereas YTHDF1 overexpression significantly enhanced the AKT3 mRNA and protein levels. These data support that AKT3 is the direct target of YTHDF1 in esophageal cancer. The results indicated that YTHDF1 might regulate the transcription and translation of AKT3. However, the fine mechanism of YTHDF1-regulated AKT3 expression needs further investigations.

**Conclusion**

In summary, we provide extensive in vitro and in vivo evidences demonstrating that m\(^6\)A modification can regulate the progression of esophageal cancer, as featured by promoting cancer cell growth, survival and invasion. Importantly, we uncovered that FTO operated a regulatory network of m\(^6\)A modification which involves METTL14, YTHDF1 and AKT3 signaling. Thus, we present the first insight of FTO-mediated
esophageal cancer progression and speculate that targeting FTO might be an effective therapeutic strategy to treat esophageal cancer.

**Abbreviations**

ES: Esophageal cancer;

m$^6$A: N$^6$-methyladenosine;

MeRIP-seq: Methylated RNA immunoprecipitation sequencing;

RNA-seq: Transcriptomic RNA sequencing;

FTO: Fat-mass and obesity-associated protein;

IHC: Immunohistochemical;

RT-qPCR: Quantitative real-time PCR;

ESCCs: Esophageal cancer cells;

AKT3: AKT serine/threonine kinase 3;

METTL14: Methyltransferase-like 14;

WTAP: Wilms tumor 1 associated protein;

ALKBH5: Alkylation repair homolog protein 5;

YTHDF1: YTH N6-methyladenosine RNA binding protein 1;

YTHDF2: YTH N6-methyladenosine RNA binding protein 2;

IGF2BP1: Insulin like growth factor 2 mRNA binding protein 1;

IGF2BP2: Insulin like growth factor 2 mRNA binding protein 2;

USTC: University of Science and Technology of China;

ActD: Actinomycetes D;

E-cad: E-cadherin;

VIM: Vimentin;

MMP2: Matrix metallopeptidase 2;
TCGA: The Cancer Genome Atlas;
3′UTR: Three prime untranslated region;
RIP: RNA immunoprecipitation;
CDS: Coding sequence;
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
WT: Wild-type;
PVDF: Polyvinylidene fluoride

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and material
Not applicable.

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

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Authors' contributions
Youguang Pu and Cunbao Zang carried out the structure of experiments and revised the
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Figures

Figure 1

FTO expression in normal esophageal tissue and ESCCs samples. A. mRNA m6A level in five esophageal cancer cell lines and one normal esophageal cell line (HEEC) determined by MeRIP-PCR with m6A RNA Methylation Quantification ELISA kit. B. FTO was up-regulated in ESCCs compared with normal tissues (GEPIA data, Red box for tumor tissue, n = 182; gray box for normal tissue, n = 286). C. Kaplan–Meier survival analysis of patient overall survival according to FTO levels in ESCCs tissues. n = 182, High FTO = 91, Low FTO = 91, p value is 0.79. D. Real-time PCR analysis and western blotting assay of FTO expression in five esophageal cancer cell lines and one normal esophageal cell line. E. Western blotting assay of FTO expression in three paired ESCCs primary tumor samples. F. Representative image of
immunohistochemical staining by FTO antibody and quantitative measurement in human samples (n = 27), Scale bar. 50 μm.

Figure 2

Effect of FTO on ESCCs cells proliferation, migration, invasion, cell apoptosis and colonies. A. The knockdown effect of specific shRNAs (sh-FTO-1, -2 and -3) in KYSE150 cells was verified at both the mRNA (by qRT-PCR) and protein levels (by western blot). ***, p value < 0.001. B. CCK-8 assays every 24 hours showed that FTO knockdown inhibited the proliferation of KYSE150 cells versus the negative control (sh-NC). ***, p value < 0.001. C. FTO knockdown in KYSE150 cells decreased migration and invasion compared to that of the negative control (sh-NC). *, p value < 0.05; **, p value < 0.01. D. The protein levels of E-cadherin and FTO in KYSE150 cells with FTO knockdown versus the negative control (sh-NC) measured by western blot analyses. E. The levels of FTO in HEEC cells transfected with FTO-OE versus the negative control (NC-OE) measured by real-time PCR and western blot analyses are shown. n.s, no statistical significance; *, p
value < 0.05; **, p value < 0.01. F.CCK-8 assays every 24 hours showed that FTO overexpression promoted
the proliferation of HEEC cells versus the negative control (NC-OE). n.s, no statistical significance; *, p
value < 0.05; ***, p value < 0.001. G.FTO overexpression in HEEC cells increased migration and invasion
compared to that of the negative control (NC-OE). The invasive ability of the cells was assessed by
Image-Pro Plus 6.0 software. **, p value < 0.01; ***, p value < 0.001. H.Colony formation assays showed
that FTO promoted cell proliferation in KYSE150 cells treated with FTO-expressing lentivirus (FTO-OE)
and FTO knockdown (sh-FTO) versus the wide type (WT). I.FTO overexpression in HEEC cells decreased
apoptosis in Annexin V/PI staining analyzed by FACS. Quantification of apoptotic cells were plotted,
numbers represent the sum of early and late apoptotic cells.
Figure 3

Correlation analysis of FTO and METTL14. A. Kaplan–Meier survival analysis of patient overall survival according to the ratio of FTO mRNA expression to METTL14 expression levels in ESCCs tissues, the higher of the ratio, the worse the prognosis. High =127, Low FTO=91, p value is 0.79. B. Wayne diagram analysis shows that the intersection genes overexpression FTO (FTO-OE) in KYSE150 and overexpression METTL14 (METTL14-OE) in KYSE150 cells. C. CCK-8 assays every 24 hours showed that FTO knockdown inhibited the proliferation of KYSE150 cells, while METTL14 knockdown (sh-METTL14) or overexpression
(METTL14-OE) can inhibit or restore FTO function. D. FTO knockdown in KYSE150 cells decreased migration, while METTL14 knockdown (sh-METTL14) or overexpression (METTL14-OE) can inhibit or restore FTO function. The migrative ability of the cells was assessed by Image-Pro Plus 6.0 software. n.s, no statistical significance; *, p value < 0.05; **, p value < 0.01. E. FTO knockdown in KYSE150 cells decreased invasion, while METTL14 knockdown (sh-METTL14) or overexpression (METTL14-OE) can inhibit or restore FTO function. The invasive ability of the cells was assessed by Image-Pro Plus 6.0 software. n.s, no statistical significance; *, p value < 0.05; **, p value < 0.01.

**Figure 4**

Identification of potential targets of FTO in ESCCs via transcriptome-wide m6A seq and RNA-Seq assays. A and B. HEEC and KYSE150 cells were treated with FTO overexpression (FTO-OE) (A) or knockdown (sh-FTO)(B), the m6A content of the total mRNA were determined with m6A RNA Methylation Quantication Kit. C. The motif of FTO was analyzed by the RMBase V2.0 database (http://ma.sysu.edu.cn/rmbase/). D. A cluster profiler identified the enriched KEGG processes of 128 genes, which showed 1.5-fold m6A expression upregulation in ESCCs cells compared with control cells. E. Dot plot analysis of the overall
level of m6A modification in esophageal cancer cells before and after knockdown of FTO m6A modification site. F. The mRNA level of six differential genes METTL1, CAMKK1, PKN2, TGFBR1, ERBB2, AKT3 and PAK1P1 in KYSE150 cells with FTO knockdown versus the negative control (sh-NC) measured by real-time PCR. n.s, no statistical significance; *, p value < 0.05; **, p value < 0.01; ***, p value < 0.001. G. The m6A abundances on AKT3 mRNA transcripts in KYSE30 and KYSE150 cells as detected by m6A-seq are plotted using Integrative genomics viewer (IGV). The y axis shows sequence read number, blue boxes represent exons, and blue lines represent introns. Reduction of m6A modification in specific regions of AKT3. H. The interaction of FTO and AKT3 was analyzed using the GEPIA database. I. Detection of m6A methylation levels in AKT3 by MeRIP-PCR with m6A RNA Methylation Quantification ELISA kit. J. Schematic representation of positions of m6A motifs within AKT3 mRNA and the 3'UTR mutation (GGAC to GGCC) of pmirGLO vector to investigate the m6A roles on AKT3 expression. pmirGLO-WT-3'UTR or pmirGLO-Mut1/2-3'UTR reporters were transfected into KYSE150 cells with FTO knockdown versus the negative control (sh-NC), then the relative luciferase activity was measured.

**Figure 5**

AKT3 is a critical target of FTO that mediate ESCCs cell growth, survival, and invasion. A. The levels of AKT3 in HEEC cells transfected with FTO-OE versus the negative control (NC-OE) measured by real-time PCR and western blot analyses are shown. *, p value < 0.05. B. The levels of AKT3 in KYSE150 cells with FTO knockdown versus the negative control (sh-NC) measured by real-time PCR and western blot analyses are shown. **, p value < 0.01. C. The mRNA level of AKT3 in KYSE150 cells with FTO knockdown versus the negative control (sh-NC) measured by real-time PCR.
knockdown and then treated with Actinomycete D (6 μg/ml) in 0, 2, 4, 6, 8 hours measured by real-time PCR. D. The knockdown effect of specific siRNAs (si-AKT3-1, -2 and -3) in KYSE150 cells was verified at both the mRNA (by qRT-PCR) and protein levels (by western blot). n.s, no statistical significance; *, p value < 0.05; **, p value < 0.01. E. The protein levels of Vimentin and AKT3 in KYSE150 cells with AKT3 overexpression versus the negative control (NC-OE) measured by western blot analyses. F. CCK-8 assays every 24 hours showed that AKT3 knockdown inhibited the proliferation of KYSE150 cells versus the negative control (sh-NC). ***, p value < 0.001. G. AKT3 knockdown in KYSE150 cells decreased migration and invasion compared to that of the negative control (si-NC). The invasive ability of the cells was assessed by Image-Pro Plus 6.0 software. *, p value < 0.05; **, p value < 0.01.

Figure 6

The functions of FTO and AKT3 on esophageal cancer cells are mutually restricted A. The relative AKT3 mRNA expression level in KYSE150 cells with FTO knockdown lentivirus (sh-FTO, mcherry), AKT3 expressing lentivirus (AKT3-OE, ZSGreen) and FTO knockdown adds AKT3 expressing lentivirus (sh-FTO+AKT3-OE) measured by qRT-PCR analyses. B. The protein levels of FTO and AKT3 in KYSE150 cells with FTO knockdown lentivirus (sh-FTO), FTO knockdown adds AKT3 expressing lentivirus (sh-FTO+AKT3-OE) measured by western blot analyses. C. CCK-8 assays showed that AKT3 knockdown inhibited the proliferation of KYSE150 cells versus the negative control (sh-NC). D. The relative proliferation rate of KYSE150 cells with different treatments measured by CCK-8 assays. E. The invasive ability of the cells was assessed by Image-Pro Plus 6.0 software.
FTO+AKT3-OE) and AKT3 expressing lentivirus (AKT3-OE) measured by western blot analyses. C. CCK-8 assays every 24 hours showed that FTO knockdown (sh-FTO) inhibited the proliferation of KYSE150 cells, while AKT3 overexpression (AKT3-OE) or FTO knockdown adds AKT3 overexpression (sh-FTO+AKT3-OE) can restore FTO function. D. The wound healing of KYSE150 cells with FTO knockdown lentivirus (sh-FTO), AKT3 expressing lentivirus (AKT3-OE) and FTO knockdown adds AKT3 expressing lentivirus (sh-FTO+AKT3-OE) for 48h were recorded (left) and quantitatively analyzed (right). E. FTO knockdown in KYSE150 cells decreased migration compared to that of the negative control (sh-NC), while AKT3 overexpression (AKT3-OE) or FTO knockdown adds AKT3 overexpression (sh-FTO+AKT3-OE) can restore FTO function. F. FTO knockdown in KYSE150 cells decreased invasion compared to that of the negative control (sh-NC), while AKT3 overexpression (AKT3-OE) or FTO knockdown adds AKT3 overexpression (sh-FTO+AKT3-OE) can restore FTO function. G. FTO knockdown in KYSE150 cells decreased colony formation compared to that of the negative control (sh-NC), while AKT3 overexpression (AKT3-OE) or FTO knockdown adds AKT3 overexpression (sh-FTO+AKT3-OE) can restore FTO function.

Figure 7

Binding verification of AKT3 gene with m6A reader. A.RIP assays in KYSE150 cells using 3xFlag, 3xFlag-YTHDC1, 3xFlag-YTHDC2, 3xFlag-YTHDF1, 3xFlag-YTHDF2 and 3xFlag-YTHDF3 plasmid and Anti-Flag
antibody. The western blots analyzed in the left showed that AKT3 interacts with YTHDC1 and YTHDF1 in KYSE150 cells. The expression of AKT3 analyzed by real-time PCR results of RIP assays are shown in the right top. The results of agarose electrophoresis of the cDNA PCR products are shown in the right bottom. n.s, no statistical significance; *, p value < 0.05; **, p value < 0.01; ***, p value < 0.001. B.Pull down assays in KYSE150 cells were transfected with biotinylated AKT3 sense probe and antisense probe (50 μl streptavidin beads were washed once with RPD buffer, then 10 μl of sense robe and 10 μl of antisense probe were added, and incubated for overnight at 4 °C). Then cells were collected for the biotin-based pull-down assay. YTHDC1 expression levels were analyzed by real-time PCR analysis and western blotting. n.s, no statistical significance; **, p value < 0.01. C.Pull down assays in KYSE150 cells were transfected with biotinylated AKT3 sense probe and antisense probe (50 μl streptavidin beads were washed once with RPD buffer, then 10 μl of sense robe and 10 μl of antisense probe were added, and incubated for overnight at 4 °C). Then cells were collected for the biotin-based pull-down assay. YTHDF1 expression levels were analyzed by real-time PCR analysis and western blotting. **, p value < 0.01; ***, p value < 0.001. D.YTHDF1 was up-regulated in ESCCs compared with normal tissues (GEPIA data, Red box for tumor tissue, n = 182; gray box for normal tissue, n = 286). E.There was no significant expression change of YTHDC1 in ESCCs compared with normal tissues (GEPIA data, Red box for tumor tissue, n =182; gray box for normal tissue, n =286). F.The real-time PCR analyzed in the top showed that the expression of YTHDF1 and AKT3 decreased with YTHDF1 overexpression (YTHDF1-OE) and the agarose electrophoresis of the PCR products also shown. The western analyzed in the bottom showed that the expression of YTHDF1 and AKT3 decreased with YTHDF1 overexpression (YTHDF1-OE). G.The real-time PCR analyzed in the top showed that the expression of YTHDF1 and AKT3 increased with YTHDF1 knockdown (sh-YTHDF1) and the agarose electrophoresis of the PCR products also shown. The western analyzed in the bottom showed that the expression of YTHDF1 and AKT3 increased with YTHDF1 knockdown (sh-YTHDF1).

Figure 8

FTO and AKT3 promote esophageal cancer invasion in vivo. A. The effect of FTO and AKT3 on tumor formation, tumor weight and tumor volume change in a nude mouse KYSE150-derived xenograft model. Representative images of tumors from FTO knockdown (sh-FTO) and AKT3-expressing lentivirus (AKT3-OE) versus the negative control sh-NC and NC-OE, respectively (n = 3 for each group). B. IHC (VIM, E-cadherin and MMP2)-stained paraffin-embedded sections obtained from sh-NC, sh-FTO, NC-OE and AKT3-OE KYSE150-derived xenografts when the tumor volumes were about 100mm3 for each group. C. KYSE150 WT, FTO knockdown (sh-FTO)-KYSE150, AKT3-expressing lentivirus (AKT3-OE)-KYSE150, and FTO knockdown+AKT3-expressing lentivirus (sh-FTO+AKT3-OE)-KYSE150 cells were injected into the nude mice by tail vein injection. Representative images of metastatic lung tumors and the H&E staining results were shown (left), and the number of lung tumors was quantitatively analyzed (right). D. The levels of epithelial mesenchymal transition (EMT) related proteins (VIME-cadherin,MMP2, Snail and N-cadherin) in KYSE150 cells with FTO knockdown (sh-FTO), FTO expressing lentivirus (FTO-OE), AKT3
knockdown (sh-AKT3), AKT3 expressing lentivirus (AKT3-OE), FTO knockdown+AKT3-expressing
tlentivirus (sh-FTO+AKT3-OE) and FTO-expressing lentivirus +AKT3 knockdown (FTO-OE+sh-AKT3) versus
the negative control (NC-OE) measured by western blot analyses.

Supplementary Files

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- Supplementarymaterials.pdf