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IRF3-mediated lncRNA FTX promotes cell proliferation, migration, invasion and suppresses cell apoptosis in oral squamous cell carcinoma by up-regulating FCHSD2 via miR-708-5p

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ABSTRACT

In recent years, researches into the molecular mechanisms of oral squamous cell carcinoma (OSCC) have improved greatly but effective targeted therapies remain elusive. More and more evidence has referred to long non-coding RNAs (lncRNAs) as modulators of carcinomas development. As a novel lncRNA, five prime to Xist (FTX), as reported before, is overexpressed in a variety of cancers. In the present study, we sought to unclose the impacts of FTX and its molecular mechanism in OSCC. Related gene expression levels were disclosed by qRT-PCR and we found that FTX was notably overexpressed in OSCC. The biological functions of FTX in OSCC were measured by functional assays. The results displayed that depletion of FTX hinderedOSCC cell migratory, invasive and proliferative abilities, but promoted cell apoptotic levels. The relationship among interferon regulatory factor 3 (IRF3), FTX, microRNA-708-5p (miR-708-5p) and FCH and double SH3 domains 2 (FCHSD2) was determined by several mechanism assays, from which we discovered that FTX activated by IRF3 regulated FCHSD2 expression by sponging miR-708-5p. Rescue experiments showed that FTX motivated OSCC development by modulating miR-708-5p/FCHSD2 axis. In summary, FTX was an oncogene in OSCC and might provide new insights into OSCC treatment.

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Introduction

Oral squamous cell carcinoma(OSCC) is considered to be fatal in many cases(1). It accounts for about 3% of malignant diseases in humans. In recent years, its incidence has been increasing among young people, with more than 500,000 newly diagnosed tumors around the world every year (2). The currently available treatment strategies include surgical resection of malignant tissue and a combination of radiation and chemotherapy, but the survival rates remain low (3, 4). Despite many innovative therapeutic strategies to combat OSCC, further research into the molecular mechanisms of OSCC is needed (5). Therefore, in order to improve the treatment and the early diagnosis rate of OSCC, identifying new high-sensitivity specificity of OSCC markers has important clinical significance for OSCC patients.

Long non-coding RNAs (lncRNAs) are defined to be transcripts with over 200nts in length and limited protein-coding ability. A large number of studies have disclosed that lncRNAsact as a key modulator of various biological processes in cancers (6). Besides, there is a lot of evidence suggesting that lncRNAs are abnormally expressed in OSCC and affect OSCC tumorigenesis and progression (7). For example, Niu et al have proposed that HOXA11-AS is an oncogene in OSCC (8). Fang et al have discovered that UCA1 participates in the modulation of proliferation and cisplatin sensitivity of OSCC via targeting the miR-184/SF1 axis (9). Zhang et al have validated that LEF1-AS1 can serve as a biomarker in OSCC (10). As a common lncRNA, FTX has been investigated in multiple cancers, such as colorectal cancer, hepatocellular carcinoma, gastric cancer and so on (11-13). Nevertheless, the role of FTX has not been explored in OSCC.

In the current study, we intended to unveil the function of FTX and further probe into the deep-going mechanism of FTX in the process of OSCC.

Materials and Methods

Cell line and cell culture

OSCC cell lines and HOK were chosen for this study. Among them, CAL-27, SCC-4 and SCC-9 OSCC cell lines were acquired from ATCCwhile TCA-8113 and HOK cell lines were commercially attained from Huatuo Biotechnology Co., Ltd. (Shenzhen, China). CAL-27 and HOK were kept in DMEM. TCA-8113 was maintained in RPMI-1640 Medium. SCC-4and SCC-9were incubated in DMEM: F-12 Medium. All the mediums were supplemented with10%FBSexcept that RPMI-1640 Medium was added with 20% FBS.

Cell transfection

Sh-FTX#1/2 and its negative controls (sh-NC) were structured, collected and purified using ultracentrifugation. Plasmid for FCHSD2 up-regulation (pcDNA3.1-FCHSD2) miR-708-5p inhibitor, mimic-miR-708-5pand the relevant negative controls were synthesized by Sargon Biotech. Lipofectamine 2000 was applied for the transfection experiments.

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The extraction of RNA and qRT-PCR

Total RNAs from OSCC cells were extracted by TRIzol reagent commercially obtained from Invitrogen. A UV-2401 spectrophotometer was adopted to determine RNA concentrations. Relative gene expression was calculated utilizing a $2^{-\Delta\Delta Ct}$ algorithm and normalized to GAPDH and U6, which acted as internal controls for miRNA and mRNA respectively.

CCK-8

OSCC cells, subsequent to transfection with miR-708-5p were fixed in a 96-well plate, with 2000 cells in each well. CCK-8 reagent was placed into each well after 48h transfection. They were kept at 37℃ in an incubator with 5% CO_2 for 24h, 48h, and 72h. A microscope was applied to count cell numbers. At 490nm, a microplate reader was used to determine the absorbance. All experiments were repeated 3 times.

Colony formation experiment

Briefly, cultured cells were placed into 6-well plates for further incubation. After 2 weeks, cells were treated with ethanol for fixation, followed by being dyed with 0.1% crystal violet. After that, stained cell colonies were calculated through manual counting. All experiments were repeated 3 times.

Transwell assay

OSCC cells were cultured using a transwell chamber system (24-well plates, Corning) (14). They were all placed into the upper chamber at 37℃ for 24h in an incubator humidified with 5% CO_2. All assays were repeated thrice.

Flow cytometry analysis

The percentage of apoptotic cells under various treatments was measured utilizing a flow cytometric assay. All the cells were transfected for 48h and 96h respectively and then cultivated in the medium at the concentration of 2×10^5 cells/2 mL in the dark at 4℃ for 15 min. All experiments were repeated 3 times.

TUNELexperiment

OSCC cells were treated with a cell toxin and fixed in 4% PFA. Next, the cells were washed twice in PBS and enhanced with 0.1% Triton X-100 for cell permeability, followed by being incubated in the dark with the TUNEL reaction mixture. After the incubation, the cells were washed in PBS and with clean water twice respectively. Lastly, a fluorescence microscope was employed to attain the images.

RNA pull-down experiment

The miR-708-5p sequences covering FTX or FCHSD2 wild-type (Wt) or mutant-type (Mut) binding sites were obtained and biotinylated. The biotin-labeled probes were then mixed with cellular proteins and Invitrogen beads. The beads are then harvested and washed. Then the precipitated RNA was eluted, purified and detected by qRT-PCR. All experiments were repeated 3 times.

RIP

OSCC cells were subjected to lysis utilizing RIP a lysis buffer. Subsequently, cell extracts were cultured with magnetic beads coupled with anti-Argonaute 2 (anti-Ago2) or control anti-IgG. The cell extract was incubated at 4℃ for 6h. Subsequent to the purification, RNA was measured using RT-qPCR to remove the globin. All experiments were repeated 3 times.

Chromatin immunoprecipitation (ChIP)

500 million cells were subjected to fixation at room temperature in 1% formaldehyde for 30 min. After that, the DNA was cut size of 500 ~ 1000bp by ultrasound technology. Immunoprecipitation of the chromatin with biotin-labeled IRF3 antibody was subsequently performed, with the anti-lgG being negative control. All assays were repeated 3 times.

Luciferase reporter assay

The sequences of FTX promoter region with the binding sites (Wt or Mut) was subcloned into the pGL3 vector, followed by transfection with pcDNA3.1/IRF3 or the empty pcDNA3.1vector into OSCC cells. The pmirGLO-FTXWt/Mutant pmirGLO-FCHSD2 3'-UTR-Wt/Mut were respectively formed by the insertion of the sequences of FTX or FCHSD2 mRNA 3'-UTRwith Wt and Mutsites of miR-708-5p into pmirGLO vector. NC mimics and miR-708-5p mimics later were co-transfected into OSCC cells along with the reporter gene. After 48h, the luciferase activity was unveiled by the Dual-Luciferase Reporter Gene Assay Kit commercially attained from Beyotime. All experiments were repeated 3 times.

Mice xenograft models

Nude, female mice were purchased from the School and Hospital of Stomatology, Jilin University. SCC-9 cells which were transfected with sh-NC were subjected to injection into three mice, and the rest were injected with FTX-depleted SCC-9 cells. After 14 days, the growth of tumors, every 4 days, was recorded. After about a month, the solid tumors were collected and the mice were sacrificed. This study used guidelines strictly and was in accordance with the School and Hospital of Stomatology, Jilin University animal care requirements.

Bioinformatics analysis

Specific miRNA regulated by lncRNA was forecasted by GEPIA(http://gepia.cancer-pku.cn/index.html),TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) and StarBase (http://starbase.sysu.edu.cn/). HumanTFBD (http://bioinfo.life.hust.edu.cn/HumanTFDB#!) was used to predict transcription factors.

Statistical analysis

The data in experiments were analyzed utilizing SPSS 21.0 software and presented as mean ± SD. Student's t-test or ANOVA was applied for difference comparison. Additionally, all the experiments were independently carried out thrice. When the P-value was lower than 0.05, it was regarded to be statistically significant.

Results

FTX expression is high in OSCC cells and promotes OSCC development

Firstly, FTX expression level in OSCC cells was unco-

vered by qRT-PCR. It was very clear that FTX was evidentlyup-regulated in OSCC cellsrelative to human normal oral keratinocytes (HOK) (Fig. 1A). Next, we transfected sh-FTX#1 and sh-FTX#2 into SCC-9 and CAL-27 cells to reduce the expression of FTX, with sh-NC as the negative control (Fig. 1B). It was unclosed by CCK-8 outcomesthat SCC-9 andCAL-27 cellviability was decreased significantly when FTX was silenced (Fig. 1C). Similarly, cell colony formation was inhibited due to depletion of FTX (Fig. 1D). Subsequently, OSCC cellmigration and invasion were unearthedby transwell assay. After transfection with sh-FTX#1 and sh-FTX#2, OSCC cell migration and invasion were observable limited.(Fig. 1E-F). Moreover, flow cytometry analysis manifested that FTX reduction promoted the apoptotic rate of OSCC cells (Fig. 1G). In summary, FTX is up-regulated and facilitates the progression of OSCC.

Down-regulation of FTX hampers tumor growth *in vivo*

To detect whether FTX promoted OSCC tumorigenicity in vivo, we carried out animal experiments. After transfection of sh-FTX#1 and sh-FTX#2, SCC-9 cells were injected into mice. From the results, we observed that in comparison with the control group, tumor growth was notably inhibited in the sh-FTX#1 group and sh-FTX#2 group (Fig. 2A-B).

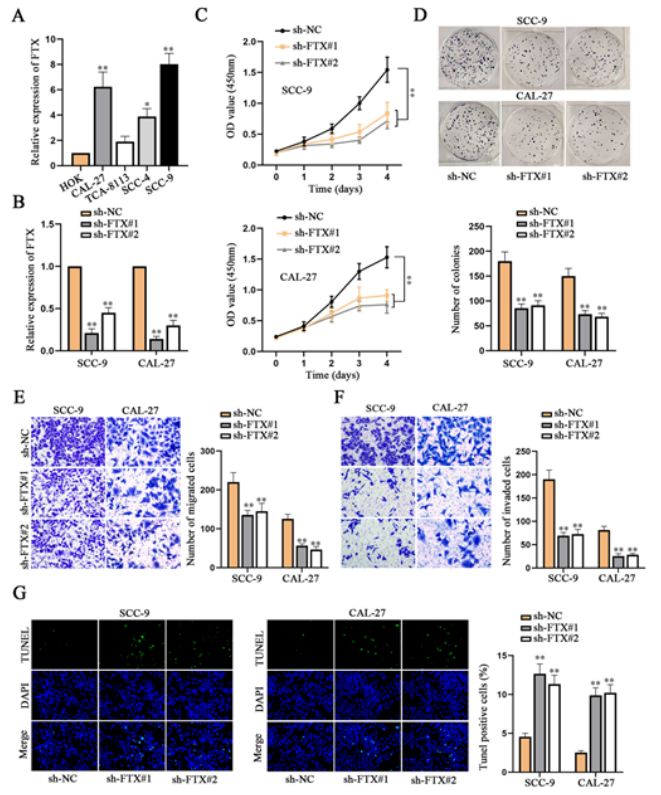

Figure 1. FTX expression is high in OSCC cells and promotes OSCC development. (A) The expression levels of FTX in OSCC cell lines (CAL-27, TCA-8113, SCC-4 and SCC-9) and normal human normal oral keratinocytes cells (HOK) were detected by qRT-PCR. (B) qRT-PCR examined the efficiency of sh-FTX#1/2. (C-D) Proliferation in SCC-9 cells and CAL-27 cells were appraised by CCK-8 and colony formation assays. (E-F) Cell migration and invasion were assessed by transwell assays. (G) The apoptosis rate was detected by flow cytometry analysis.* P<0.05, ** P<0.01.

IRF3 promotes the transcription of FTX in OSCC cells

It is known that transcription factors are always involved in the dysregulation of lncRNAs. As the HumanTFBD database predicted, we selected the top 10 transcription factors with high scores (Fig. 3A). Through qRT-PCR, we found that there are four dramatically up-regulated transcription factors (EZH2, RAD21, TP53, IRF3) in OSCC cells (Fig. 3B). Among the four transcription factors, FTX could only be regulated by IRF3 (Fig. 3C). Hence, we selected IRF3 for further study. According to the data from the JASPAR database, we identified two potential binding sites between the FTX promoter and IRF3 transcription factor. Luciferase reporter assay was utilized to validate the definite binding site. We mutated the first part, the second part, and both parts, respectively. The results show that the binding force is strongest when no mutation occurs at the binding site (Fig. 3D). Subsequently, a ChIP assay was performed to further testify that the enrichment of IRF3 in FTX promoter in OSCC cells (Fig. 3E). All the above results suggested that IRF3 activates the transcription of FTX in OSCC.

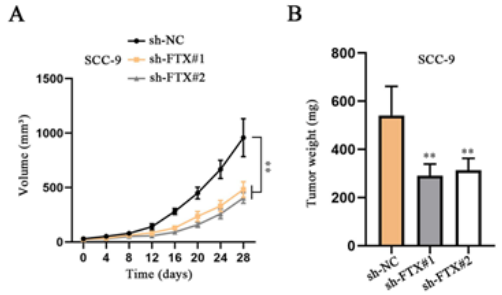

Figure 2. Down-regulation of FTX inhibits tumor growth in vivo. (A) Differences in tumor volume and tumor growth rate in the sh-NC group and sh-FTX#1/2 group. (B) The weight of tumors in different groups. ** P<0.01.

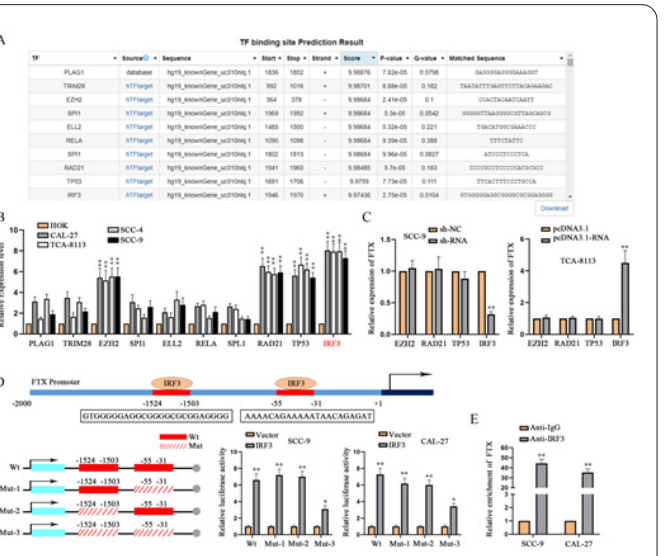

Figure 3. IRF3 promotes the transcription of FTX in OSCC cells. (A) Bioinformatics analysis of ten highly expressed transcription factors. (B) qRT-PCR detected the expression levels of ten transcription factors in OSCC cells. (C) The expression of FTX when selecting 4 transcription factors was knocked down or overexpressed. (D) Predicted binding sites and the verification of binding sites by the implementation of luciferase reporter assays. (E) CHIP was performed to validate the binding. * P<0.05, ** P<0.01.

FTX sponges miR-708-5p

Then, we conducted a subcellular fractionation assay and found that FTX majorly existed in OSCC cell cytoplasm (Fig. 4A). Therefore, we speculated that FXT might play the role of competitive endogenous RNA (ceRNA). We used Starbase and screened out9 miRNAs with the highest correlation, and qRT-PCR outcomes revealed that miR-144-5p, miR-708-5p and miR-142-5p expression levels in OSCC cells were low (Fig. 4B). RNA pull-down assay demonstrated that instead of miR-144-5p and miR-142-5p, only miR-708-5p could be pulled down by biotinylated FTX probe (Fig. 4C). In the StarBase, the binding sites between FTX and miR-708-5p were forecasted(Fig. 4D). The results of Ago2 RIP assay verified the interaction between FTX and miR-708-5p (Fig. 4E). From the results of RNA pull-down assay, we found that FTX was significantly pulled down by biotinylated miR-708-5p-Wt probe (Fig. 4F). And the binding force between FTX and miR-708-5p was then validated by the luciferase reporter assay (Fig. 4G). Thus, FTX competitively sponges miR-708-5p in OSCC cells.

FCHSD2 is targeted by miR-708-5p and is negatively modulated by miR-708-5p

We then attempted to investigate the downstream target gene of miR-708-5p in OSCC. Through intersection with all the mRNAs that had binding sites with miR-708-5p in the StarBase database and the obviously up-regulated

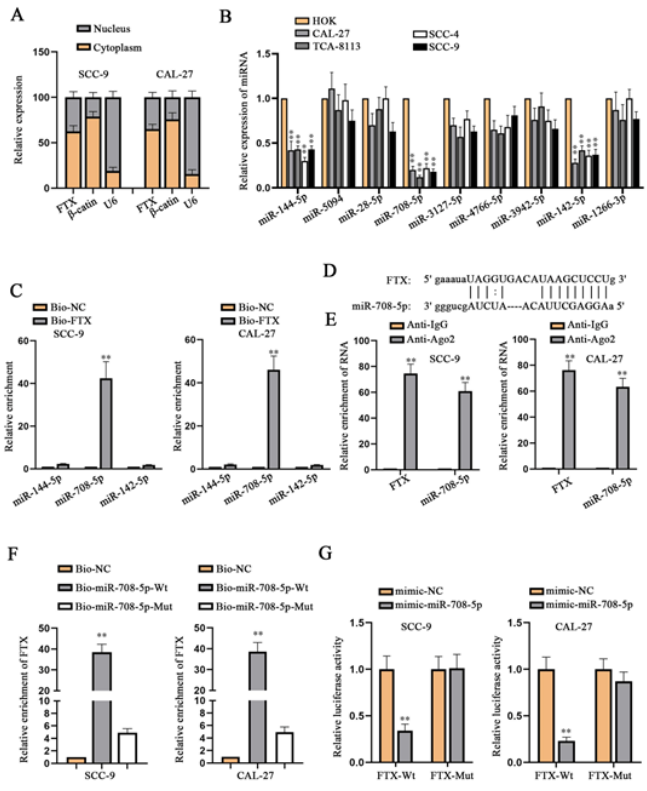

Figure 4. FTX sponges miR-708-5p. (A) The location of FTX in OSCC cells was judged. (B) The expression levels of 9 miRNAs in OSCC cell lines were measured by qRT-PCR. (C) The enrichment of 3 miRNAs when pulled down by a biotinylated FTX probe. (D) Binding sites between miR-708-5p and FTX. (E) The binding force was validated by Ago2 RIP. (F) Binding sites were detected by RNA pull-down. (G) Binding sites were examined by luciferase reporter assays. ** P<0.01.

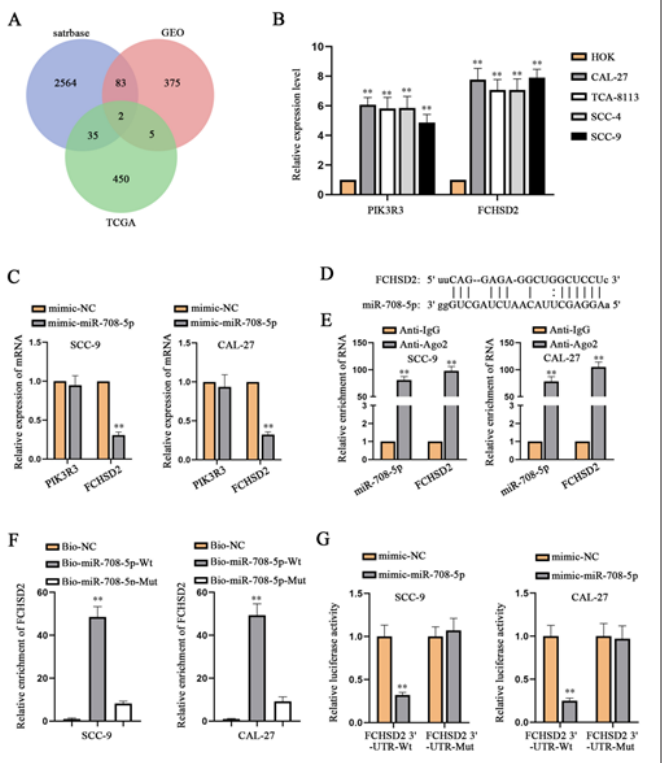

Figure 5. FCHSD2 is a target gene of miR-708-5p and is negatively regulated by miR-708-5p. (A) Two mRNAs were chosen by bioinformatics analysis. (B) qRT-PCR assays measured the expression levels of two mRNAs in OSCC cell lines and normal cell lines. (C) The expression of 2 mRNAs when miR-708-5p was overexpressed. (D) Binding sites between FCHSD2 and miR-708-5p. (E) The binding force was detected by Ago2 RIP. (F) Binding sites were verified by RNA pull-down assay. (G)Binding sites were examined by luciferase reporter assays. ** P<0.01.

mRNAs in TCGA and GEO, we got two mRNAs (PIK3R3 and FCHSD2) (Fig. 5A). It was obvious that FCHSD2 were highly expressed in OSCC cells (Fig. 5B). Moreover, when miR-708-5p was up-regulated, FCHSD2 instead of PIK3R3 was notably decreased (Fig. 5C). We predicted the binding sites between FCHSD2 and miR-708-5p in StarBase (Fig. 5D). Due to the binding site, we carried out an Ago2 RIP experiment to verify the combination between FCHSD2 and miR-708-5p. The results uncovered that there is a strong binding force between them (Fig. 5E). Similarly, as manifested by RNA pull-down assay, miR-708-5p could bind to FCHSD2 (Fig. 5F). Then, we performed luciferase reporter assay to demonstrate that mimic-miR-708-5p transfection could significantly reduce the luciferase activity in the FCHSD2 3'-UTR-Wt group (Fig. 5G). To be concluded, FCHSD2 is targeted by miR-708-5p and is negatively modulated by miR-708-5p.

Inhibition of miR-708-5p or overexpression of FCHSD2 rescues the inhibition ofFTX knockdown on OSCC development

At last, in order to probe into the FTX/miR-708-5p/FCHSD2 axis in OSCC progression, we conducted rescue experiments. SCC-9 cells and CAL-27 cells were utilized subsequent to transfection with sh-NC, sh-FTX#1, sh-FTX#1+miR-708-5p inhibitor, and sh-FTX#1+pcDNA3.1-FCHSD2 respectively. As displayed in Fig. 6A-B, the decreased proliferative capacity of OSCC cells due to FTX inhibition could be recovered by miR-708-5p inhibitor or

FCHSD2 up-regulation. Transwell assays clearly showed that FTX-induced suppressed cell migration and invasion in SCC-9 cells and CAL-27 cells were significantly reversed by miR-708-5p down-regulation or FCHSD2 overexpression (Fig. 6C-D). Moreover, FTX depletion facilitated cell apoptosis while miR-708-5p inhibitor or FCHSD2 overexpression countervailed this effect(Fig. 6E). In summary, through miR-708-5p/FCHSD2 axis, lncRNA FTX participated in OSCC progression.

Scientific concept map describes the functional mechanism of FTX in OSCC cells

We concluded that IRF3-activated FTX promoted OSCC cell progression sequestering sponging miR-708-5p to promote FCHSD2 expression (Fig. **7**).

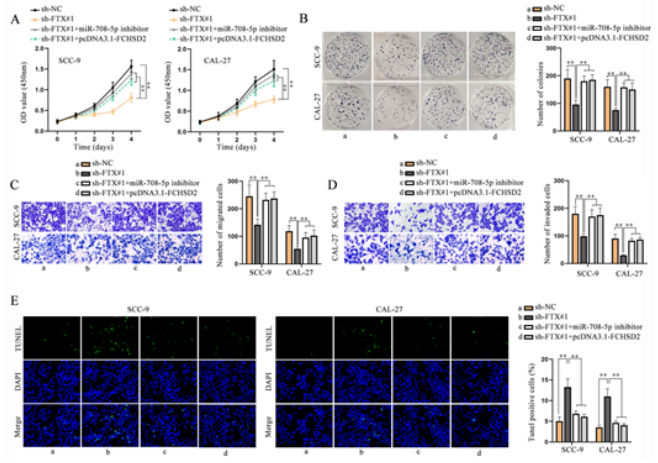

Figure 6. Inhibition of miR-708-5p or overexpression of FCHSD2 rescues the inhibition of FTX knockdown on OSCC development. (A-B) CCK-8 and colony formation assays were conducted to appraise the proliferative capacity in SCC-9 cells and CAL-27 cells. (C-D) Migration and invasion in SCC-9 cells and CAL-27 cells were assessed by transwell assays. (F) Apoptosis rates in SCC-9 cells and CAL-27 cells were detected by flow cytometry assay. ** P<0.01

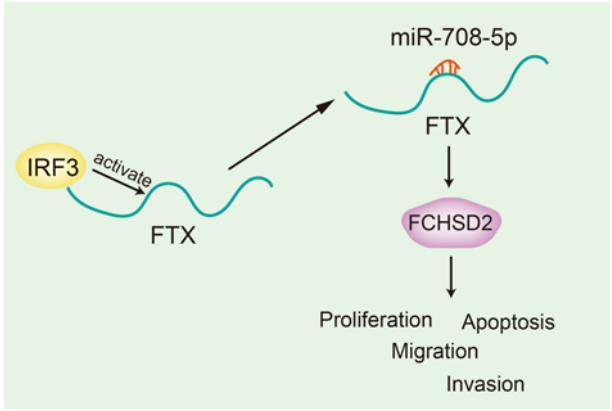

Figure 7. Scientific concept map describes the functional mechanism of FTX in OSCC cells.

Discussion

OSCC is the most frequent oral malignancy and affects many people every year(15). Recently, a large amount of evidence has been unclosed that lncRNA is associated with the development of OSCC (7, 16). In this study, we identified a promising new biomarker lncRNA FTX in OSCC. FTX has been determined to be an oncogene in diverse cancers and promoted cancer progression, such as colorectal cancer, gastric cancer, osteosarcoma and so on (11, 13, 17). Consistent with these findings, we found that silencing of FTX obviously hindered cell progression in OSCC. Besides, tumor growth was also inhibited by the depletion of FTX. These results confirmed the carcinogenic effect of FTX and confirmed that FTX was a positive regulator of OSCC progress.

Bioinformatics analysis showed that transcription factor IRF3 combined with FTX promoter and propelledFTX transcription. Furthermore, many studies have reported that lncRNAs can play the role of ceRNAs through competitive binding with miRNAs, thus regulating the expression of target mRNAs. This led us to further investigate the possible mechanism of FTX in OSCC. The main distribution of FTX in OSCC cell cytoplasm further validated that FTX might serve as a ceRNA. MiR-708-5p has been identified to be a biomarker in multiple cancers (18-20). According to bioinformatics analysis, it was unearthed that miR-708-5p might have a putative binding site with FTX. A series of experiments also proved that miR-708-5p was sponged by FTX. Bioinformatics analysis also predicted that FCHSD2 was a promising target of miR-708-5p, which was then attested by mechanism assays. Furthermore, it was unmasked by rescue experiments that miR-708-5pimpairmentor FCHSD2overexpression could offset the influential role of FTX knockdown in OSCC development.

In summary, we elucidated the role of FTX in OSCC and verified the functions of the FTX/miR-708-5p/FCHSD2 axis in OSCC development. The findings of this study contribute to a more in-depth understanding of the occurrence and development of OSCC, as well as provide a new theoretical basis for OSCC treatment.

To sum up, all the above results indicate that FTX is overexpressed in OSCC, which aggravates migration, invasion, proliferation and represses apoptosis of OSCC cells. Transcription factor IRF3 activates FTX transcription and mediates its up-regulation in OSCC cells. Moreover, FTX sequesters miR-708-5p and enhances FCHSD2 expression, thereby promoting OSCC cell progression and hindering OSCC cell apoptosis. The above conclusion suggests that FTX may be a potential therapeutic target in OSCC.

Conflict of interest statement

None declared.

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