Original Paper

Long Noncoding RNA FER1L4 Suppresses Tumorigenesis by Regulating the Expression of PTEN Targeting miR-18a-5p in Osteosarcoma

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Key Words

Long non-coding RNA • FER1L4 • PTEN • miR-18a-5p • Osteosarcoma

Abstract

Background/Aims: Novel long non-coding RNA Fer-1-like protein 4 (FER1L4) has been reported to play crucial regulatory roles in tumor progression. However, its clinical significance and biological role in osteosarcoma (OS) is completely unknown. The aim of the present study was to investigate the role of FER1L4 in OS progression and the underlying mechanism.

Methods: We analyzed the expression levels of FER1L4 in tissues of OS patients and cell lines via quantitative RT-PCR and RT-PCR. The effect of FER1L4 on cell proliferation, colony formation, migration and invasion was analyzed by cell counting kit-8 (CCK-8), colony formation, wound healing and transwell invasion assay, respectively. Novel targets of FER1L4 were selected through a bioinformatics soft and confirmed using a dual-luciferase reporter system and qPCR. To detect the role of FER1L4 *in vivo* tumorigenesis, tumor xenografts were created.

Results: We found that the expression of FER1L4 was significantly downregulated in OS tissues and cell lines; moreover, low expression of FER1L4 was associated with advanced tumor-metastasis (TNM) stage, lymph node metastases, and poor overall survival. Functional assays showed that upregulation of FER1L4 significantly inhibited OS cell proliferation, colony formation, migration, and invasion *in vitro*, as well as suppressed tumor growth *in vivo*. Assays performed to determine the underlying mechanism, indicated that FER1L4 interacted directly with miR-18a-5p. Subsequently, we found that FER1L4 significantly increased PTEN expression, a known target of miR-18a-5p, in OS cells. Furthermore, PTEN was found to be

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down-regulated, and positively correlated with FER1L4 in OS tissues. **Conclusion:** These findings suggest that FER1L4, acting as a competing endogenous RNA (ceRNA) of miR-18a-5p, exerts its anti-cancer role by modulating the expression of PTEN. Thus, FER1L4 may be a novel target for the prevention and treatment of OS.

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Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor with high morbidity, in children and young adults [1]. With advances in both, diagnosis and treatment, the five-year survival rate in non-metastatic OS patients has reached 60%–70% [2]. However, the prognosis of OS patients with metastasis remains poor [3]. Although several studies have been carried out on alterations in the genome and transcriptome, the mechanism underlying the pathogenesis and progression in OS remains largely unclear. Thus, there is an urgent need to explore the mechanism of OS progression, which might contribute to the development of effective strategies in the diagnosis, treatment, and prognosis of OS patients.

Long non-coding RNAs (lncRNAs) are a type of non-protein-coding RNAs, comprising more than 200 nucleotides in length, with no protein-coding capacity [4, 5]. Increasing evidence suggests that lncRNAs play crucial roles in tumorigenesis and the development of cancer by regulating proliferation, metastasis, cell cycle progression, cell growth, and apoptosis [6–8]. In OS, some lncRNAs have been reported to be involved in the pathogenesis of OS as oncogenes or tumor suppressor genes [9–10], which indicated that lncRNAs serve as a diagnostic marker or therapy target for OS.

Fer-1-like protein 4 (FER1L4), a newly identified tumor-associated lncRNA, has been reported to be downregulated in gastric cancer [11], hepatocellular carcinoma [13], colon cancer [14], and endometrial carcinoma [15], and acts as a tumor suppressor in these cancers. However, the clinical significance and biological role of FER1L4 in OS is still unclear. Therefore, in this study, we aimed to investigate the clinical significance of FER1L4, its biological function, and the underlying mechanism in OS. Our finding will provide new insights into the molecular function of FER1L4 in OS, and provide a new diagnostic marker and therapy target for the disease.

Materials and Methods

Tissue samples

OS tumor tissues and adjacent normal bone tissues were obtained from 48 patients with OS, who received surgical treatment from 2013 to 2014 at China-Japan Union Hospital of Jilin University (Changchun, China). None of the patients received neoadjuvant therapy or radiotherapy before surgery. All tissues were immediately frozen and stored in liquid nitrogen, until use. This study was approved by the hospital's ethics committee and all patients signed a written consent form. Clinic and pathological data were acquired from the medical records (Table 1).

Cell culture

Four human OS cell lines, Saos-2, U2OS, MG-63, and 143B and normal human osteoblasts, hFOB 1.19, were purchased from American Type Culture Collection (Manassas, USA). The cells were grown in Dulbecco's modified Eagle's medium medium (DMEM; Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 units/ml of penicillin and streptomycin (Invitrogen, CA, USA) in a humidified incubator at 37 °C, under 5% CO₂.

Table 1. Correlation between clinicopathological features and FER1L4 expression in 48 patients with osteosarcoma

Variables	No. of cases	FER1L4 expression		P value
		High (n %)	Low (n %)	
Age(years)				
<20	28	13(46.4)	15 (53.6)	p>0.05
≥20	20	9(45.0)	12(55.0)	
Gender				
Male	24	12(50.0)	12(50.0)	p>0.05
Female	24	10(41.7)	14(58.3)	
TNM stage				
I-II	37	21(56.8)	16(43.2)	p<0.01
III-IV	11	1(9.1)	10(90.9)	
Tumor size				
<5 cm	29	14(48.3)	15(51.7)	p>0.05
≥5 cm	19	8(42.1)	11(57.9)	
Metastasis				
No	38	22(57.9)	16(42.1)	p<0.01
Yes	10	0(0)	10(100)	

Plasmid, miRNA mimics, and cell transfection

The coding sequence of FER1L4 was amplified by PCR using human liver cDNA (Invitrogen), and cloned into pCDNA3.1 vector; it was referred to as pFER1R4. miR-18a-5p mimic and its corresponding negative control mimic (miR-NC) were bought from Genechem Corporation (Shanghai, China). Cells were seeded into 24-well plates, and grown to ~80% confluency, and transfected with the above-mentioned plasmid or its corresponding mimic, using Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer's protocols.

Real-time RT-PCR

Trizol Reagent (Invitrogen) was used to isolate total RNA from tissues or cultured cells, according to the manufacturer's instructions. RNA samples were reverse-transcribed into cDNA, using PrimeScript RT Master Mix Perfect Real Time (Takara, Dalian, China) with Oligo (dT18) RT primers. Quantitative real-time PCR (qPCR) was performed using SYBR Green Master Mix (Applied Biosystems). GAPDH was used as an internal control. For detection of miR-18a-5p, miRNA was isolated from tissues or cells using miRNeasy kit (Qiagen, German), following manufacturer's instructions. cDNA was synthesized using miScript RT kit (Qiagen) and quantified by quantitative real-time PCR analysis experiments using miScript SYBR Green PCR Kit (Qiagen), according to manufacturer's instructions. U6 was used as internal control. All reactions were performed on the ABI 7900 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) in, at least, triplicates prepared for each sample. All primers are enlisted in Table 2. Relative expression of miRNA or mRNA was determined after normalizing to the internal control by using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation and colony formation assay

Cell viability was determined with Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan). Briefly, 1×10^3 transfected cells were seeded into a 96-well plate with quadruplicate repeat for each condition. At different time points (24 h, 48 h, and 72 h), 10 μ l CCK-8 solution was added into each well for an additional 4 h at 37°C. Absorbance was measured at wavelength of 450 nm, in a microplate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA).

For the colony formation assay, 1,000 transfected cells were grown in six-well plates (in triplicate), and allowed to adhere and colonize for 2 weeks. Thereafter, cell colonies were fixed in 96% ethanol for 10 min and stained with 1% crystal violet for 5 min. Cell colonies were imaged and counted.

Cell cycle assay

Cell cycle was determined by flow cytometry, following the instructions of the cell cycle staining kit (KeyGEN, Nanjing, China) on a FACS Aria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle was analyzed using CellQuest software 3.1 (BD Biosciences, San Jose, CA, USA).

Wound healing assays and cell invasion assays

Cell migration was determined by wound-healing assays. Briefly, transfected cells were cultured overnight in 6-well plates (5×10^4 cells per well). These cultures were then scratched to create an artificial wound using a sterile plastic micropipette tip. Cells were further cultured with serum-free medium for 24 h. At 0 and 24 h after the scratch, photographs were taken using an X71 inverted microscope (Olympus, Tokyo, Japan).

Cell invasion was measured using Matrigel invasion assays. Briefly, 1×10^5 transfected cells in 100 μ l free-serum DMEM medium were seeded into the upper BD BioCoat Matrigel Invasion Chamber (BD Biosciences), while 600 μ l medium, containing 20% FBS, was added to the lower chamber to stimulate cell invasion. After incubation for 48 h, cells on the upper membrane surface were removed using a cotton swab and the cells that had traversed the membrane were fixed with 70% ethanol for 10 min and stained with 0.1% crystal violet for 15 min. These invasive cells that had moved across the membrane were photographed and counted in five randomly selected fields using a light microscope (magnification, 200 x, Tokyo).

Table 2. Real time PCR primers used for mRNA expression analysis. Abbreviations: F, forward; mRNA, messenger RNA; PCR, polymerase chain reaction; R, reverse

Target gene	Prime(5'-3')
U6	F- TCCGATCGTGAAGCGTTC R- GTGCAGGTCGCCAGGT
miR-18a-5p	F- TCCGAGATAGACGTGATCT R- GTGCAGGTCGCCAGGT
FER1L4	F- CCGTGTCTCTGTGCTG R- GGCAAGTCTCTGTCAG
PTEN	F- -GTTTACCGGCACTCAAA R- CCCCACTTTAGTCTTCACT
GAPDH	F- AAGGAGTGGTGGTGAAG R- -AATCCTTCTCATTTTGG

Bioinformatics prediction and luciferase reporter assay

To predict the binding sites between FER1L4 and miR-18a-5p, a publicly available algorithm (starBase v2.0) was used. We constructed the luciferase reporter vectors with wild-type (WT) binding sites and mutant-type (MT) binding sites of FER1L4 and PTEN (pmirGLO-FER1L4-Wt, pmirGLO-FER1L4-Mt). The U2OS cells were cotransfected with the luciferase reporter vectors along with miR-18a-5p mimics or miR-NC. Forty-eight hours after transfection, Luciferase activity was determined using the Dual-Luciferase Reporter System (Promega, Madison, WI) following the manufacturer's protocols. The results were represented as the ratio of firefly luciferase activity to Renilla luciferase activity.

Western blot

Primary antibodies against PTEN and GAPDH were purchased from Santa Cruz Biotechnology (USA). For western blot analyses, total proteins were extracted from cells and tissues using RIPA Lysis Kit (Beyotime, Jiangsu, China), and concentration of protein was measured using a BCA Protein Assay Kit (Bio) following the manufacturer's instructions. An amount of 40 µg total protein, per sample, was separated by 8% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Billerica, MA, USA). Subsequently, the membrane was blocked for 2 h using 5% skim milk, and incubated with the appropriate primary antibodies at 4°C overnight. Thereafter, the membrane was incubated with horseradish peroxidase-labeled secondary antibodies (goat anti-mouse IgG, Santa Cruz Biotechnology) at 37°C for 1 h. Protein bands were observed with the enhanced chemiluminescence kit (WesternBrightTina, Advanta Corporation, CA). Quantitative analysis of the blots was performed using ImageJ software (Bethesda, MD, USA).

Tumor xenograft model

Twenty 6-week-old male BALB/c nude mice, weighing 18–20 g, were obtained from the Experimental Animal Center of Jilin university (Changchun, China) and kept under specific pathogen-free conditions in this center. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University.

Equal numbers of U2OS cells (2×10^6) transfected with pFER1L4 plasmid or pcDNA3.1 were suspended in 100 µl serum-free DMEM and injected subcutaneously into the right posterior flanks of the mice (n=10 each group). Tumor volume (V) was monitored and calculated according to the formula: $V = 0.536 \times L \times W^2$, measuring tumor length (L) and width (W) every 7 days for 28 days. Mice were euthanized by cervical dislocation 28 days after injection, and tumor tissues were removed and weighed; Ki-67 expression was studied using immunohistochemistry. In addition, FER1L4 expression was measured in tumor tissues by qRT-PCR.

Immunohistochemistry

Immunohistochemistry (IHC) assays were performed to detect Ki-67 expression in tumor tissues as previously described [16]. Rabbit anti-Ki-67 primary antibody was bought from Cell Signaling Technology (Beverly, MA, USA). The IHC staining was evaluated by two pathologists and immunohistochemical scoring was calculated as previously described [16].

Statistical analysis

Data are presented as mean ± standard deviation (SD) from at least three independent experiments, and was analyzed using version 19 SPSS statistical software (SPSS, Chicago, IL, USA) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were analyzed using the Student's *t* test or one-way analysis of variance (ANOVA). Spearman's correlation coefficient was used to assess correlations. Overall survival and recurrence-free survival was analyzed by Kaplan-Meier survival analysis and compared using the log-rank test. For all statistical analyses, *P* values <0.05 were considered statistically significant and are indicated as follows: **p* <0.05 and ***p* <0.01.

Results

FER1L4 expression was down-regulated in OS tissues and correlated with poor prognosis

To investigate the role of FER1L4 in OS progression, we first examined the relative expression level of FER1L4 in OS tissues, in comparison to the adjacent normal bone tissues. qRT-PCR data showed that FER1L4 expression was lower in OS tissues than that in corresponding normal bone tissues (Fig. 1A). In addition, we also determined FER1L4 expression level in four human OS cell lines (Saos-2, MG-63, U2OS and 143B) and in normal human osteoblasts (hFOB 1.19). As shown in Fig. 1B, the expression of FER1L4 was obviously downregulated in the OS cell lines, compared to the hFOB 1.19 cells. We then investigated the correlation between clinicopathological characteristics and FER1L4 expression. The data revealed that FER1L4 expression was closely associated with TNM stage ($p < 0.01$) and lymph node metastasis ($p < 0.01$). Nevertheless, FER1L4 expression level was not associated with age, gender, and tumor size (Table 1). To further explore the involvement of FER1L4 in the survival of patients with OS, we analyzed the correlation between the FER1L4 expression level and the survival of OS patients. Kaplan-Meier analyses indicated that patients with low levels of FER1L4 had statistically significantly worse overall and progression-free survival, relative to the high FER1L4 expression group ($p \leq 0.05$, Fig. 1C and D). These results indicated that low FER1L4 expression was related to poor prognosis, and FER1L4 might be involved in OS progression.

Overexpression of FER1L4 suppresses OS cell proliferation, colony formation, migration, and invasion in vitro

To further explore the biological function of FER1L4 in OS, U2OS cells were transfected with pFER1L4 plasmid and blank vector plasmid (pCMV3.1). Thereafter, the biological functions of FER1L4 were determined in vitro. Satisfactory transfection efficiency was obtained at 48 h post-transfection and confirmed by qRT-PCR (Fig. 2A). CCK-8 assays showed that overexpression of FER1L4 significantly suppressed cell proliferation in U2OS cells (Fig. 2B). Consistent with these results, colony formation assays revealed that overexpression of FER1L4 significantly reduced colony formation of U2OS cells (Fig. 2C). To

Fig. 1. FER1L4

expression was downregulated in OS tissues and correlated with poor prognosis. (A) qRT-PCR analysis of FER1L4 expression in OS tissues and corresponding normal bone tissues. (B) qRT-PCR analysis of FER1L4 expression in OS cells (Saos-2, U2OS, MG-63 and 143B), compared with hFOB1.19. (C, D) Kaplan-Meier plots representing probabilities of progression-free and overall survival in OS

patients based on FER1L4 expression level. High FER1L4 expression ($\geq$ median value of expression) and low FER1L4 expression ($<$ median value of expression) were used to define groups. * $p < 0.05$ and ** $p < 0.01$.

Fig. 2. Overexpression of FER1L4 suppresses OS cell proliferation, colony formation, migration, and invasion in vitro. (A) qRT-PCR analysis of FER1L4 mRNA expression in U2OS cells transfected with pFER1L4 plasmid and blank vector (pCDNA3.1). (B-F) Cell proliferation, colony formation, cycle arrest, migration, and invasion were determined in U2OS cells, transfected with pFER1L4 plasmid pCDNA3.1. * $p < 0.05$ and ** $p < 0.01$.

investigate the influence of FER1L4 on migration and invasion of OS cells, wound healing and transwell invasion assays were performed. We found that overexpression of FER1L4 significantly suppressed migration and invasion of U2OS cells (Fig. 2D and E). These results suggested that FER1L4 inhibits OS cell proliferation, migration, and invasion.

Upregulation of FER1L4 inhibits tumor growth in vivo

To explore whether FER1L4 could also regulate tumorigenesis *in vivo*, we performed a xenograft tumor growth assay. The FER1L4 transfected cells and the vector transfected cells were injected into two sets of nude mice, and tumor growth was measured. We observed that xenograft tumor growth was slower in the FER1L4 injected group than the blank vector injected group (Fig. 3A). At 28 days post-injection, the mice were sacrificed, and tumor tissues were removed and weighted. We found that both, average tumor size and weight, were decreased significantly in the FER1L4 injected group compared to the blank vector control group (Fig. 3B and C). Moreover, we observed that Ki-67 protein, a proliferation marker, was decreased in the FER1L4 injected group (Fig. 3D). Finally, we also observed that tumor tissue has a higher expression of FER1L4 in the FER1L4 injected group than in the vector control group (Fig. 3E). These results suggested that upregulation of FER1L4 suppresses tumor growth *in vivo*.

Fig. 3. Upregulation of FER1L4 inhibits tumor growth in vivo. (A) Tumor volumes were measured and calculated every week, for 4 weeks. (B) Shown are images of xenograft tumors at 28 days post injection. (C) Shown are weight of xenograft tumors at 28 days post injection. (D) qRT-PCR analysis of FER1L4 mRNA expression in xenograft tumors. (E) Ki-67 protein expression was detected in xenograft tumors by immunohistochemistry. * $p < 0.05$ and ** $p < 0.01$.

FER1L4 directly interacts with miR-18a-5p

Recent evidence suggest that lncRNAs recruit miRNAs to regulate gene expression through an miRNA-mediated mechanism [18]. Herein, the candidate miRNAs of FER1L4 were predicted using starBase v2.0. Amongst target miRNAs, miR-18a-5p was selected for the study, based on its biological functions in various cancers [19, 20]. There is a complementary binding site between FER1L4 and miR-18a-5p (Fig. 4A). To gain more insight into the relationship between FER1L4 and miR-18a-5p, luciferase reporter assay was performed. As shown in Fig. 4B, compared to the other groups, cotransfection with miR-18a-5p mimic and pmirGLO-FER1L4-Wt significantly reduced the luciferase activity of U2OS cells ($p < 0.01$). The data indicated that miR-18a-5p directly binds to FER1L4 binding sites and downregulate the luciferase activity. In addition, we found that transfection with miR-18a-5p mimics, significantly downregulated the expression level of FER1L4 in OS cells ($p < 0.01$; Fig. 4C); similarly the upregulation of FER1L4 significantly decreased the expression of miR-18a-5p in U2OS cells ($p < 0.01$; Fig. 4D). We also determined the expression of miR-18a-5p in the 48 OS tissues samples, and analyzed the correlation between miR-18a-5p and FER1L4. It was found that miR-18a-5p expression was upregulated (Fig. 4E), and was inversely correlated with FER1L4 expression in the OS tissues ($r = -0.723$; Fig. 4F). We performed rescue experiments to determine the effect of miR-18a-5p on the role of FER1L4 in inhibiting OS cell proliferation, migration, and invasion. The results of the CCK-8 assay, wound healing, and Transwell invasion assay showed that upregulation of FER1L4 inhibited proliferation, migration, and invasion, and these inhibition effects were partly abolished by the introduction of miR-18a-5p in U2OS cells (Fig. 4G-I). These data suggest that FER1L4 directly interacts with miR-18a-5p in OS cells and tissues.

Fig. 4. FER1L4 directly interacts with miR-18a-5p. (A) Bioinformatic tools located the complementary binding sequence of miR-18a-5p and 3'UTR binding site (Wt) and mutant type (Mt). (B) Luciferase reporter assay was performed in U2OS cells transfected with miR-18a-5p or negative control mimic and pmirGLO-FER1L4-Wt or pmirGLO-FER1L4-Mt reporter plasmids. (C) qRT-PCR analysis of FER1L4 mRNA expression in U2OS cells transfected with miR-18a-5p mimics or negative control mimic (miR-NC). (D) qRT-PCR analysis of miR-18a-5p expression in U2OS cells transfected with pFER1L4 plasmid or blank vector. (E) qRT-PCR analysis of miR-18a-5p expression in OS tissues and the corresponding normal bone tissues. (F) A negative relationship between the expression of FER1L4 and miR-18a-5p in OS tissues was observed (n=48). (G-I) Cell proliferation, migration and invasion were determined in U2OS cells transfected with pFER1L4 plasmid/pcDNA3.1 plasmid with or without miR-18a-5p mimic. *p<0.05 and **p<0.01.

Associated role of FER1L4, miR-18a-5p, and PTEN in OS proliferation, migration, and invasion

PTEN, a well-known tumor suppressor, has previously been shown as a target of miR-18a-5p in lymphoma, carcinoma and OS [21, 22]. To explore the relationship among FER1L4, miR-18a-5p and PTEN, the U2OS cells were transfected with miR-18a-5p mimics or pFER1L4 plasmid, and PTEN expression was examined in the transfected cells at both, mRNA and protein levels. Compared to the control groups, cells transfected with pFER1L4 plasmid showed significantly increased PTEN expression at both, mRNA and protein levels in U2OS cells (Fig. 5A, B). However, the cells that were transfected with miR-18a-5p, exhibited the opposite trend (Fig. 5A-B). Moreover, we also determined the expression of PTEN mRNA in the 48 OS tissue samples and analyzed the correlation between PTEN mRNA and FER1L4. We observed that the expression of PTEN mRNA was significantly decreased in OS tissues (Fig. 5C), and was positively correlated with the expression of FER1L4 ($r=0.643$; Fig. 5D). Therefore, these results indicated an associated role of FER1L4, miR-18a-5p, and PTEN in OS progression.

Fig. 5. Associated role of FER1L4, miR-18a-5p and PTEN on OS proliferation, migration, and invasion. (A) qRT-PCR analysis of miR-181-5p expression in U2OS cells transfected with miR-18a-5p mimic/miR-NC or pFER1L4 plasmid/ pcDNA3.1 plasmid. (B) qRT-PCR analysis of PTEN mRNA expression in U2OS cells transfected with miR-18a-5p mimic/miR-NC or pFER1L4 plasmid/ pcDNA3.1 plasmid.

(C) qRT-PCR analysis of PTEN mRNA expression in OS tissues and the corresponding normal bone tissues. (D) A positive relationship between the expression of FER1L4 and PTEN in OS tissues was observed (n=48). *p < 0.05 and **p < 0.01.

Discussion

The development and progression of OS occurs through a series of sequential and complex steps that involves the aberrant expression of several genes [23, 24]. Some lncRNAs are proven to play crucial roles in the origin and development of OS, as oncogenes or tumor suppressors [9, 10]. Therefore, there is an urgent need to explore novel lncRNAs in OS for developing diagnostic markers and therapy targets. In this study, we investigated the role of FER1L4 in OS progression.

FER1L4, a novel long non-coding RNA, has been reported to be involved in initiation and development of many cancers by regulating cancer cell proliferation, migration, apoptosis, and invasion [11–15]. For example, Wu et al. reported that FER1L4 could exert a tumor suppressive role in liver cancer, at least partially, through suppression of miR-106a-5p expression [11]. Qiu et al. showed that FER1L4 suppresses cancer cell proliferation and cell cycle by regulating PTEN expression in endometrial carcinoma [15]. Xia et al. found that lncRNA FER1L4 exerts tumor suppressive roles in gastric cancer by acting as a ceRNA of miR-106a-5p to regulate the expression of PTEN [12]. However, the exact role of FER1L4 in OS progression and the underlying mechanism remain unclear. Therefore, in the present study, we performed an in-depth investigation of the involvement of FER1L4 in OS. To the best of our knowledge, we, for first time, have demonstrated that FER1L4 expression is downregulated in OS samples and cell lines. Moreover, low expression of FER1L4 was associated with advanced TNM stage, lymph node metastases, and poor overall survival. Functional assays demonstrated that upregulation of FER1L4 in OS cells significantly inhibited cell proliferation, colony formation, migration, and invasion *in vitro*, and suppressed tumor growth *in vivo*. These results suggest that FER1L4 functions as a tumor suppressor in OS progression.

lncRNAs are known to be involved in a series of cellular biological processes by acting as ceRNAs or molecular sponges, to modulate the effect of miRNAs [17, 18]. To investigate the anti-cancer mechanism of lncRNA in OS, we predicted the candidate miRNAs of FER1L4, using the starBase v2.0 software. Among the target miRNAs, miR-18a-5p was selected as the study object, based on its biological function in various cancers [19, 20]. miR-18a-5p,

a member of the miR-17-92 cluster, has been reported to be upregulated, and functions as an oncogene in OS progression [25-27]. We further confirmed by luciferase reporter assay and qRT-PCR, that FER1L4 interacts directly with miR-18a-5p, both in OS cell lines as well as in tissues. In addition, our data also showed that there is a negative correlation between FER1L4 and miR-18a-5p in OS tissues. Importantly, we found that upregulation of FER1L4 inhibited proliferation, migration, and invasion, and these inhibitory effects were partly abolished by the introduction of miR-18a-5p in OS cells. These studies suggest that FER1L4 functions as a ceRNA of miR-18a-5p in OS.

Phosphatase and tensin homologue (PTEN), located on chromosome 10q23, has been reported to be downregulated in OS tissues and cell lines, and low PTEN expression is associated with poor prognosis [28-30]. In addition, overexpression of PTEN significantly inhibits OS cell proliferation, migration, and invasion, and induces cell apoptosis [31-33]. Of note, the regulatory mechanism between FER1L4 and PTEN has been revealed in endometrial carcinoma [15] and gastric cancer [12]. Recently, a study showed that miR-18a-5p plays an oncogenic role by repressing PTEN expression in esophageal carcinoma [21] and colon [22]. Consistent with previous results, we found that miR-18a-5p overexpression decreased PTEN expression, while FER1L4 overexpression obviously increased PTEN expression in OS cells. Moreover, we also found that FER1L4 expression was positively correlated with PTEN in OS tissues. Our results not only corroborate previous findings, but also suggest that FER1L4/miR-18a-5p/PTEN axis is a novel target for prevention and treatment of OS.

There are several limitations in this study. Firstly, we investigated the anti-cancer function and the underlying mechanisms of FER1L4 on U2OS cells. The role of FER1L4 in other OS cell lines needs to be revealed to cross-verify the effects of FER1L4 in OS. Secondly, the function of FER1L4 was evaluated through overexpression of FER1L4 in a gain-of-function model. Loss-of-function studies via downregulation of FER1L4 in OS cells are needed to verify our findings.

Conclusion

Our data demonstrate that FER1L4 is down-regulated in OS tissues and cell lines, and associated with TNM stage, lymph node metastasis, and poor overall survival. Overexpression of FER1L4 repressed cell proliferation, colony formation, migration, and invasion *in vitro*, as well as impaired tumor growth *in vivo*. In addition, FER1L4 exercised its tumor suppressor role partially, by targeting miR-18a-5p/PTEN pathway. Our findings suggest that FER1L4 is a potential therapeutic target for the treatment of OS.

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Disclosure Statement

The authors declare that they have no competing interests.

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