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Increased expression of lncRNA FTH1P3 promotes oral squamous cell carcinoma cells migration and invasion by enhancing PI3K/Akt/GSK3b/ Wnt/β-catenin signaling

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Abstract. – OBJECTIVE: Growing evidence indicated that long noncoding RNAs (lncRNAs) played important roles in tumor initiation and progression. In this study, we aimed to investigate the expression pattern, clinical significance, and biological function of lncRNA ferritin heavy chain 1 pseudogene 3 (FTH1P3) in oral squamous cell carcinoma (OSCC).

PATIENTS AND METHODS: We evaluated the expression levels of FTH1P3 in OSCC tissues, adjacent normal tissues and cells with quantitative Real-time polymerase chain reaction. Kaplan-Meier curves and multivariate Cox proportional models were used to study the impact on clinical outcome. Effect of FTH1P3 on the proliferation migration and invasion of OSCC cells was estimated by MTT, wound-healing, and transwell assays. Western blot was performed to investigate the effects of FTH1P3 on expression of PI3K/Akt/GSK3β-related molecules, and Wnt/β-catenin signaling components.

RESULTS: The expression level of FTH1P3 was significantly upregulated in OSCC tissues and cell lines. Higher expression of FTH1P3 in OSCC tissue was associated with T classification, N classification and TNM stage. Furthermore, Kaplan-Meier survival analysis showed that prognosis of patients with low FTH1P3 expression was much better than that of those with high expression. Cox regression analysis showed that FTH1P3 expression was an independent prognosis-predicting factor for OSCC patients. Loss-function assay showed that knockdown of FTH1P3 significantly suppressed the proliferation, migration and invasion of OSCC cells. Mechanistically, we found that knockdown of FTH1P3 significantly reduced the activation of PI3K/Akt/GSK3β/Wnt/β-catenin signaling.

CONCLUSIONS: We demonstrated that FTH1P3 acted as a tumor promoter by regulating PI3K/Akt/GSK3β/Wnt/β-catenin signaling and could represent a novel prognostic marker in OSCC patients.

Key Words:
LncRNA, FTH1P3, Prognosis, Tumorigenesis, Wnt/β-catenin signaling, PI3K/Akt/ GSK3β signaling.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common oral cavity cancer affecting about 500,000 new cases worldwide each year, and the incidence of new cases indicates a continuing rise in developing countries[1,2]. Several risk factors, such as smoking, alcohol consumption and HPV infection, can affect the genesis of OSCC[3]. Despite of improvements in surgery, radiotherapy and chemotherapy, the survival rate of OSCC patients has not significantly improved in the past decades[4,5]. The poor prognosis of OSCC individuals is largely due to the inability to diagnose at early stage and tumor metastasis[6]. Thus, it is urgent to explore the potential mechanisms involved in OSCC metastasis, to identify reliable biomarkers of OSCC, and to be able to predict the prognosis of this disease. Long noncoding RNAs (lncRNAs) are a newly emerged class of noncoding RNA containing more than 200 nucleotides that are widely transcribed in the genome[7]. Recently, evidence indicates that lncRNAs play important

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roles in the regulation of multiple biological processes, including proliferation, metastasis, differentiation and carcinogenesis[8,9]. Notably, lncRNAs were found to function as either oncogene or anti-oncogene to participate in the pathogenesis and development of many kinds of cancers[10,11]. For instance, lncRNA ANRIL, a well-known lncRNA, was found to promote proliferation and metastasis of several tumors, including non-small cell lung cancer[12], bladder cancer[13] and thyroid cancer[14]. lncRNA UCA1, a well-studied lncRNA, was reported to exert oncogenic functions in various tumors, such as breast cancer[15], osteosarcoma[16] and OSCC[17]. Although several lncRNAs have been identified in OSCC, the expression and function of most lncRNAs have not been investigated[18,19]. Long non-protein coding RNA ferritin heavy chain 1 pseudogene 3 (FTH1P3), a member of the ferritin heavy chain (FHC) gene family, is a newly identified lncRNA. Although previous researches have reported that FTH1P3 was highly expressed in OSCC and served as a tumor promoter, the clinical significance and potential mechanism of FTH1P3 need to be further elucidated[20]. In this study, we also found that FTH1P3 expression was significantly upregulated in OSCC. The clinical assay showed FTH1P3 levels were associated with prognosis of OSCC patients. In addition, loss-function assay revealed that knockdown of FTH1P3 significantly suppressed the proliferation, migration and invasion of OSCC cells by modulating PI3K/Akt/GSK3β/Wnt/β-catenin signal.

Patients and Methods

Patients and Specimens

134 patients with OSCC who were diagnosed, treated, and followed at the Department of Oral Medicine, Qilu Hospital, Institute of Stomatology, Shandong University between 20011 and 2013, were recruited for the present study. The samples were snap-frozen in liquid nitrogen in the operating room and stored at -80°C until extraction of total RNA. None of the patients had received radiotherapy or chemotherapy prior to surgery and all the histological diagnoses for OSCC were confirmed by two independent pathologists. The tumors were classified according to the Union for International Cancer Control TNM classification system. All 134 patients with OSCC had valid follow-up data. The overall survival (OS) was defined as the time between diagnosis and the date of death or the date last known alive. Informed written consents were obtained from all patients included in this study, which was approved by the Ethics Committee of Qilu Hospital.

Cell Culture and Transfection

Four OSCC cell lines (SNU1041, SCC25, SCC4 and SCC9) and a human normal oral keratinocyte cell line (hNOK) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Xuhui, Shanghai, China). All cells were cultured in RPMI-1640 medium (Roswell Park Memorial Institute-1640) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco Rockville, MD, USA), 100 U/ml penicillin (Meilun, Xicheng, Beijing, China), and 100 μg/ml streptomycin in culture flasks at 37°C with 5% CO_2. Knockdown of FTH1P3 was performed by transfecting cells with siRNA specifically targeting FTH1P3 (GenePharma, Pudong, Shanghai, China). Scrambled negative control siRNA (Scrambled) were purchased from Invitrogen (Carlsbad, CA, USA). SCC25 and SCC4 cells were transfected with siRNA or scramble using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested for qRT-PCR or Western blot analysis.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cultured cell lines and fresh tissue samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Complementary DNA was generated using a miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). qRT-PCR was carried out on an ABI Prism 7200 HT sequence detector (Applied Biosystems, Foster City, CA, USA), along with a Fast SYBR Green Master Mix kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instruction. The internal control for each sample used in this study was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The qRT-PCR reactions were performed in triplicate. FTH1P3: 5'-TCCATTTACCTGTGCGTGGC-3' (forward) and 5'-GAAGGAAGATTCGGCCACCT-3' (reverse). GAPDH: 5'-CTGTCCTCGCCGTCACACCG-3' (forward) and 5'-GGCATGGACTGTGGTCATGAG-3' (reverse). The qRT-PCR reactions were performed in triplicate. Fold changes in expression of each gene were calculated by

a comparative threshold cycle (Ct) method using the formula $2^{-(\Delta\Delta Ct)}$.

Cell Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to detect the growth of OSCC cells and the growth curve was delineated. 5×10^3 cells per well were seeded in triplicate with complete growth medium in a 96-well plate. A total of 20 µl of 5 mg/ml MTT solution (SigmaAldrich, St. Louis, MO, USA) was added to each well and 150 µl dimethyl sulfoxide was added then incubated for 4 h at 37°C. ELISA reader was used to measure the absorbance at 490 nm.

Wound Healing Assay

SCC25 and SCC4 cells after transfection were seeded in 6-well plates at 70% confluence and cultured until they formed a monolayer that occupied 100% of the surface area. The monolayer was scratched with a sterile 10 µl pipette tip, and the floating cells were carefully removed by phosphate-buffered saline (PBS). The scratch width after 0 and 48 hours was observed using an inverted microscope and photographed.

Transwell Assay

Transwell invasion assays were performed to determine SCC25 and SCC4 cell invasion abilities using transwell chamber (Millipore, Billerica, MA, USA). 5×10^4 cells were seeded in the upper well of the invasion chamber in Dulbecco's Modified Eagle Medium (DMEM) without serum. In the lower chamber, 0.8 ml Roswell Park Memorial Institute-1640 (RPMI-1640) with 10% fetal bovine serum (FBS) was added. After 24 h of incubation, the chambers were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet. The number of invasive cells on the lower surface of the membrane was then counted under a microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

Total proteins were extracted from OSCC cell lines and tissues using a RIPA buffer with 0.5% sodium dodecyl sulfate (SDS) in the presence of a proteinase inhibitor cocktail. The protein concentration was measured by a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein lysates were isolated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking, blots were probed with following primary antibodies including Akt, p-Akt, GSK3b, p-GSK3b and β-catenin overnight at 4°C. All antibodies were purchased from Cell Signaling (Danvers, MA, USA). Then, the membranes were washed and subsequently treated with secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein was detected with Image Acquisition using ImageQuant™ LAS 4000 (GE Healthcare Life Sciences, Haidian, Beijing, China).

Statistical Analysis

All the statistical analyses and graphics were performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA) or SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed by the two-tailed Student's *t*-test. The relationships between FTH1P3 expression and clinicopathological parameters were examined by chi-square test. Survival curves were plotted using the Kaplan-Meier method and differences in survival rates were analyzed using the log-rank test. Cox proportional hazards regression analysis was used for univariate and multivariate analyses of prognostic values. A p-value of < 0.05 was considered significant.

Results

FTH1P3 is Increased in OSCC and Correlated to Aggressive Features

In order to identify differentially expressed lncRNA between OSCC tissues and adjacent non-cancer tissues, we performed microarray analysis. As shown in Figure 1A, our results showed the 32 most significantly differentially expressed lncRNA transcripts. Among them, FTH1P3 was remarkedly up-regulated in OSCC tissues. Thus, our present study focused FTH1P3. In order to further explore the expression of FTH1P3 in OSCC tissues, we performed RT-PCT to detect the levels of FTH1P3 in OSCC samples from our hospital. As shown in Figure 1B, up-regulation of FTH1P3 was found in OSCC tissues compared with corresponding noncancerous tissues ($p < 0.01$). Four human OSCC cell lines were also tested. The OSCC cell lines showed a remarkably high expression of FTH1P3 compared to a

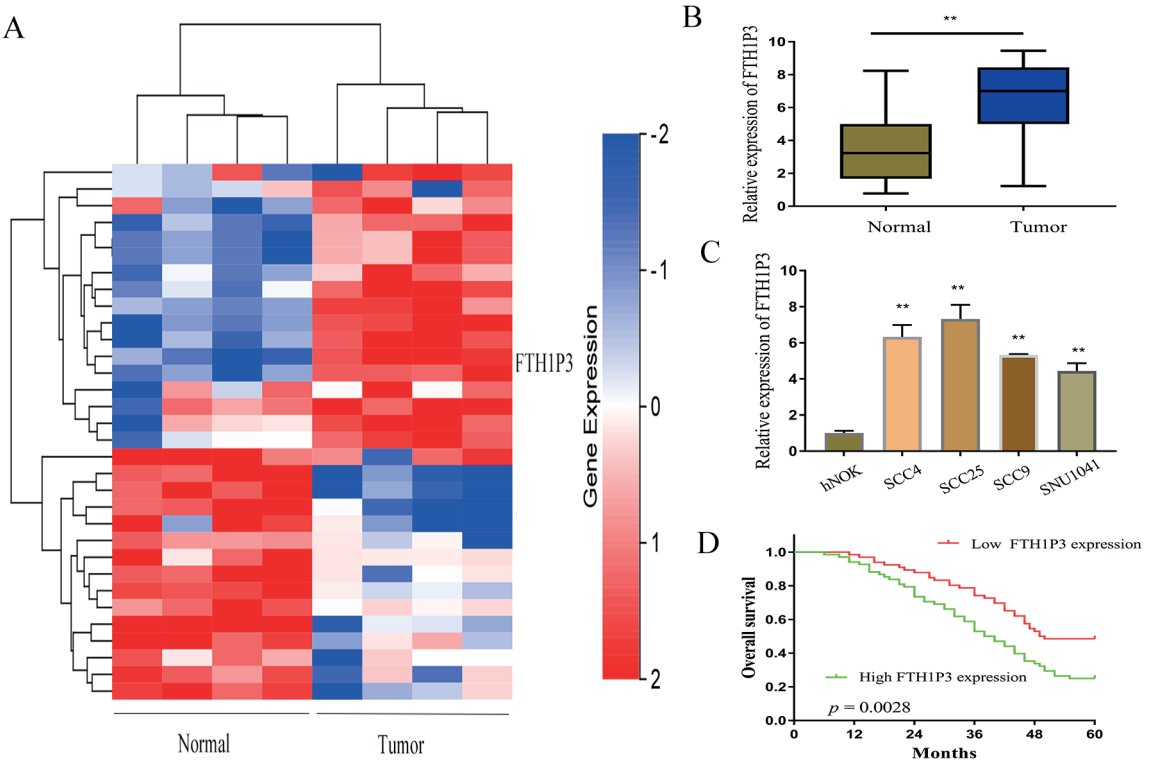

Figure 1. Relative FTH1P3 expression in OSCC tissues and its clinical significance. ***(A)*** Heat map analysis of lncRNAs expression of groups was created using a method of hierarchical clustering by GeneSpring GX, version 7.3. ***(B)*** FTH1P3 expression was analyzed in the OSCC paired sample tissues by qRT-PCR. FTH1P3 expression levels were normalized to GAPDH. ***(C)*** Relative FTH1P3 levels in OSCC cell lines (SNU1041, SCC25, SCC4 and SCC9). ***(D)*** Kaplan-Meier method with the log-rank test was used to analyze the overall survival curves of patients in high and low FTH1P31 expression groups. *$p < 0.01$, **$p < 0.05$.

human oral keratinocyte cell line hNOK (Figure 1C). In addition, we analyzed the association between the expression of FTH1P3 and clinicopathological characteristics of OSCC. As shown in Table I, we observed that FTH1P3 expression was closely correlated with T classification ($p = 0.009$), N classification ($p = 0.032$) and TNM stage ($p = 0.005$). Taken together, our results indicated that FTH1P3 expression was significantly upregulated in OSCC patients and associated with aggressive clinical features.

The Association Between FTH1P3 Expression and Overall Survival of Patients with OSCC

Whether FTH1P3 expression had prognostic potential for the overall survival of OSCC patients was investigated. During the follow-up, 85 patients were died and the median follow-up time was 343 ± 11.6 months. Using the Kaplan-Meier method and log-rank test, the overall survival of osteosarcoma patients with high FTH1P3 expression was significantly shorter than those with low FTH1P3 expression ($p = 0.0028$, Figure 1D). Subsequently, univariate and multivariate analysis using the Cox proportional hazard regression model was used to explore whether FTH1P3 expression level and other clinical parameters are independent factors for prognostic prediction in OSCC patients. As shown in Table II, we found that FTH1P3 expression, T classification, N classification and TNM stage, were associated with overall survival of OSCC patients by univariate analysis. Moreover, multivariate analysis revealed that FTH1P3 expression (HR = 3.135, 95% CI: 1.223-5.237, $p = 0.006$), as well as TNM stage, N classification and T classification, was an independent prognostic parameter indicating poor prognosis for OSCC patients.

Knockdown of FTH1P3 Suppresses OSCC Cells Proliferation, Migration and Invasion in vitro

To explore whether the level of FTH1P3 expression could affect OSCC cells tumorigenesis, we used siRNA to knockdown the FTH1P3

Table I. Association between FTH1P3 expression and clinicopathological features of human OSCC.

Clinicopathological features	Total	FTH1P3 expression		*p*-value
		High	Low	
Age				0.2877
< 60	75	35	40	
≥ 60	59	33	26	
Gender				0.617
Male	76	40	36	
Female	58	28	30	
Histology/differentiation				0.147
Well + moderate	81	37	44	
Poor	53	31	22	
T classification				0.009
T1 + T2	72	29	43	
T3 + T4	62	39	23	
N classification				0.032
Positive	55	34	21	
Negative	79	34	45	
TNM stage				0.005
I + II	75	30	45	
III + IV	59	38	21	

expression in SCC25 and SCC4, and accessed the role of FTH1P3 in OSCC proliferation and metastasis. qRT-PCR analysis showed that FTH1P3 expression was significantly downregulated in the OSCC-transfected cells compared with their respective controls ($p < 0.01$, Figure 2A), which confirmed the infection effect. Then, MTT assays revealed that knockdown of FTH1P3 significantly decreased cell viability of OSCC cell lines (SCC25 and SCC4) (Figure 2B and 2C). In addition, we performed wounding healing assays and the results showed that SCC25 and SCC4 cells in the si-FTH1P3 group had stronger wound healing ability than those in the scramble group (Figure 2D). Furthermore, invasion assay indicated that SCC25 and SCC4 cells in the si-FTH1P3 exhibited decreased invasive abilities compared with those displayed by scramble cells (Figure 2E). Taken together, our results revealed that knockdown of FTH1P3 expression markedly reduces the proliferation, migration and invasion motility of OSCC cells (Figure 3).

FTH1P3 Regulates PI3K/Akt Axis and Wnt/β-Catenin Signaling in OSCC Cells

Currently, known signaling pathways involved in the regulation of tumorigenesis include PI3K-Akt, Wnt-β-catenin, and Notch pathways, among others[21,22]. In order to explore the potential mechanism by which FTH1P3 exhibited its anti-cancer role in OSCC, our attention focused on the association between FTH1P3 and PI3K/Akt/GSK3β/Wnt/β-catenin signaling. The results of Western blot showed that knockdown of FTH1P3 significantly inhibited the activity of β-catenin, p-AKT and p-GSK3β. These data revealed that FTH1P3 could in some way regulate the activity of PI3K/Akt/GSK3β/Wnt/β-catenin signaling pathway in OSCC cells.

Discussion

OSCC is a health problem worldwide with increasing incidence and mortality rates[23]. Growing data have shown that lncRNAs are dysregulated in various cancers and might contribute to the activation and upregulation of key molecules in cancers[24]. Zhan et al[20] showed that FTH1P3 was overexpressed in OSCC tissues. However, since then, no further expression or functional data on FTH1P3 in OSCC has been reported. Furthermore, the clinical significance of FTH1P3 in OSCC has not been investigated. In this study, we detected the expression of FTH1P3 in both OSCC tissues and cell lines and the results showed that FTH1P3 was significantly up-regulated in both OSCC tissues and cell lines, confirming that FTH1P3 may serve as a tumor promoter which positively controlled OSCC progression. Clinical assay showed that FTH1P3 expression was closely correlated with

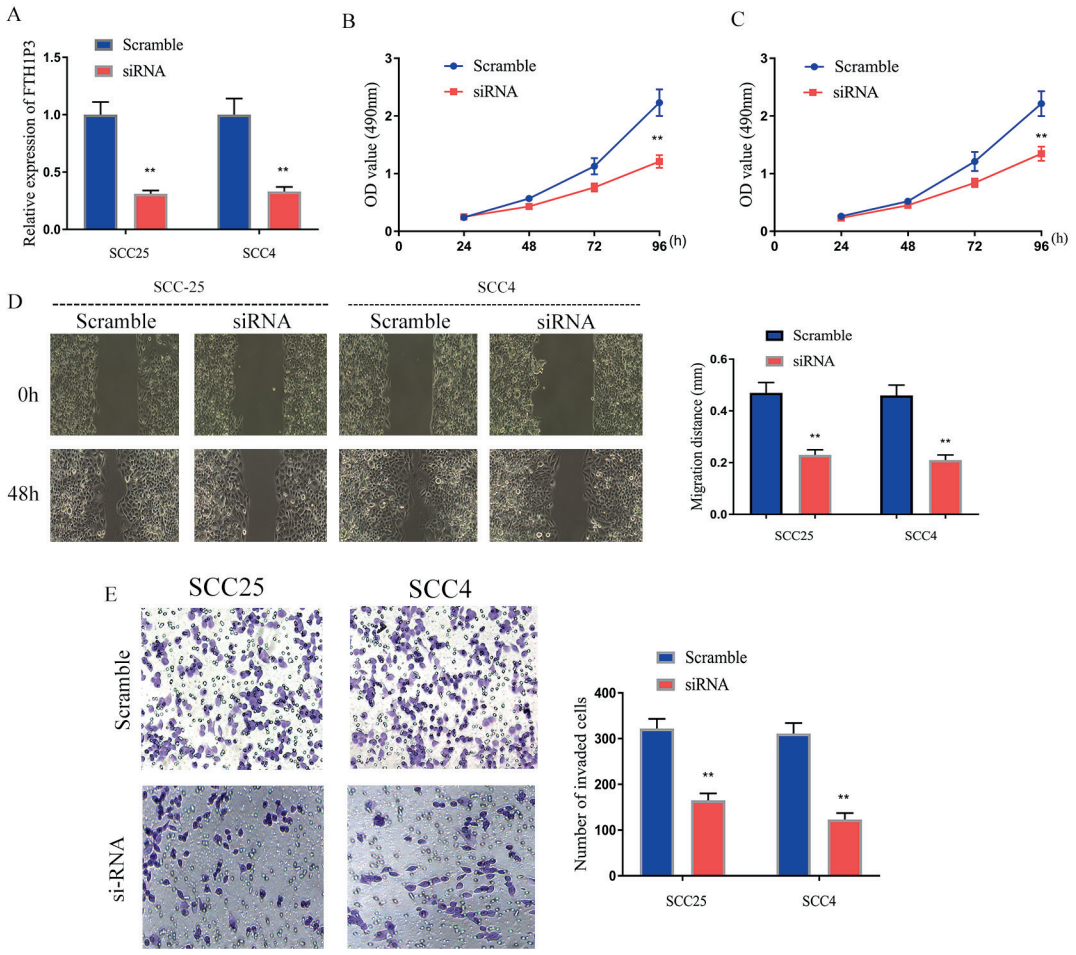

Figure 2. Knockdown of FTH1P3 inhibits proliferation, migration and invasion of SCC25 and SCC4 cells. ***(A)*** qRT-PCR analysis of FTH1P3 expression in SCC25 and SCC4 cells transfected with si-FTH1P3 and scramble. ***(B-C)*** Cell proliferation curves in SCC25 and SCC4 cells by MTT assay. ***(D)*** The wound-healing assay determined cell migration in both SCC25 and SCC4 cells. ***(E)*** Transwell invasion assay determined cell migration in both SCC25 and SCC4 cells. *$p < 0.01$, **$p < 0.05$.

Table II. Summary of univariate and multivariate Cox regression analyses of overall survival duration.

	Univariate analysis			Multivariate analysis		
Parameter	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
Age	1.332	0.765-1.885	0.327	-	-	-
Gender	1.534	0.833-2.236	0.136	-	-	-
Histology/differentiation	1.449	0.589-2.332	0.117	-	-	-
T classification	3.336	1.457-5.679	0.008	2.567	1.256-4.456	0.015
N classification	2.783	1.216-4.223	0.011	2.463	1.138-3.994	0.035
TNM stage	3.678	1.589-5.557	0.004	3.138	1.237-4.451	0.009
FTH1P3 expression	4.132	1.632-7.773	0.001	3.135	1.223-5.237	0.006

T classification, N classification and TNM stage, indicating FTH1P3 as a positive regulator in clinical progression of OSCC. Next, Kaplan-Meier analysis indicated that patients with high FTH1P3 expression lived shorter than those with low expression. Based on those findings, we further performed Cox regression analysis and the results proved that FTH1P3 may be a poor independent prognostic factor for OSCC patients. Taken together, our findings indicated

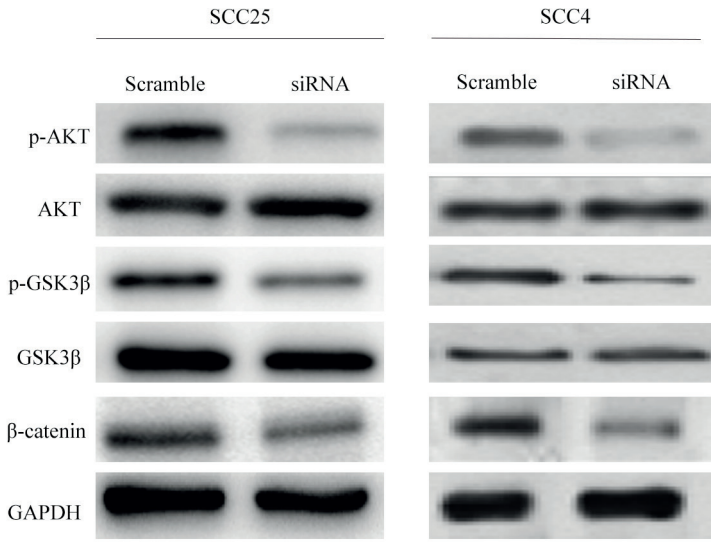

Figure 3. Knockdown of FTH1P3 inhibits the activity of GSK-3β/β-catenin signaling in SCC25 and SCC4 cell lines. The levels of PI3K/Akt/GSK3b/ Wnt/β-catenin in SCC25 and SCC4 treated with si-FTH1P3 or scramble by Western blot.

that FTH1P3 acted as an important regulator in the modulation of OSCC progression and may be considered as a novel prognostic marker for OSCC. FTH1P3, a novel noncoding RNA mapping to 2p23.3 chromosomal region, was originally detected as markedly high level in OSCC[25]. Recent studies indicated that FTH1P3 was involved in tumorigenesis because its expression is increased in several cancers. For instance, Zheng et al[26] reported that FTH1P3 was highly expressed in uveal melanoma and its overexpression promoted melanoma cell proliferation and migration miR-224-5p. Zhang et al[25] found that FTH1P3 was highly expressed in OSCC tissues by Microarray assay. Zhang et al[20] revealed that FTH1P3 expression was up-regulated in OSCC tissues and associated with a worse overall survival of OSCC patients. However, their samples are small. In addition, they did not perform Cox regression analysis. Our results further confirmed the prognostic value of FTH1P3 in OSCC. On the other hand, in their *in vitro* assay, they found that overexpression of FTH1P3 promoted OSCC cell growth by acting as a molecular sponge of miR-224-5p. In our present study, we also get the similar results that knockdown of FTH1P3 suppressed the proliferation of OSCC cells. Furthermore, we firstly explore whether FTH1P3 was involved in the regulation of metastasis. As expected, it was observed that down-regulation of FTH1P3 by siRNA significantly suppressed OSCC cells migration and invasion. Thus, our results, together with previous study, indicated FTH1P3 as an important tumor promoter in OSCC. PI3K/Akt/GSK-3β and Wnt/β-catenin are aberrant in a wide variety of cancers and have been shown to play a critical role in cancer cell proliferation, migration, invasion, and apoptosis[27,28]. Many studies[29,30] had reported that lncRNAs could exhibited their role by modulating PI3K/Akt/GSK-3β and Wnt/β-catenin in various tumors. In this study, we hypothesized that FTH1P3 may be associated with Wnt/β-catenin signaling, involving PI3K/Akt/GSK-3β signaling and cell cycle progression. In order to demonstrate this hypothesis, we down-regulated the expression of FTH1P3 by siRNA and explored the expression levels of PI3K/Akt/GSK-3β/Wnt/β-catenin-related proteins. We found that knockdown of FTH1P3 significantly suppressed the expression of p-AKT, p-GSK3β and β-catenin, indicating the tumor promoter role of FTH1P3 in OSCC by modulating PI3K/Akt/GSK-3β/Wnt/β-catenin signaling.

Conclusions

We provided evidence that high expression of FTH1P3 was significantly associated with tumor progression and decreased survival in patients

with OSCC. In addition, we first reported that FTH1P3 has the potential to suppress OSCC cells metastasis *in vitro* possibly by modulating PI3K/Akt/GSK-3β/Wnt/β-catenin signaling. These findings suggested that FTH1P3 may act as a novel prognostic marker and a potential therapeutic target for this disease.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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