

Original Article

Long non-coding RNA FTH1P3 promotes the metastasis and aggressiveness of non-small cell lung carcinoma by inducing epithelial-mesenchymal transition

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Received June 17, 2019; Accepted September 23, 2019; Epub October 1, 2019; Published October 15, 2019

Abstract: Long non-coding RNAs (lncRNAs) ferritin heavy chain 1 pseudogene 3 (FTH1P3) has been suggested to act as an oncogene in many types of human malignancy, but its role in non-small cell lung carcinoma (NSCLC) remains unknown. This study aimed to characterize the biologic functions of FTH1P3 in NSCLC and illuminate its clinical significance. The expression levels of FTH1P3 in NSCLC tissues and cell lines were detected by quantitative real-time PCR assay. The relationship of FTH1P3 expression with clinicopathologic features was evaluated by chi-square test, and its correlation with prognosis of NSCLC patients was analyzed by Kaplan-Meier method with log-rank test. Wound healing and transwell invasion assays were applied to evaluate cell migration and invasion abilities, respectively. Western blot was performed to detect the changes of epithelial-mesenchymal transition (EMT) related protein expression. The results showed that FTH1P3 was highly expressed in NSCLC tumor tissues and NSCLC-derived cell lines, and high expression of FTH1P3 was associated with advanced TNM stage and lymph node metastasis. NSCLC patients with high FTH1P3 expression had a poor overall survival relative to patients with low FTH1P3 expression. Through loss-of-function studies, FTH1P3 inhibition was demonstrated to suppress NSCLC cell migration and invasion *in vitro*. Notably, FTH1P3 knockdown could decrease expression of N-cadherin, vimentin and Snail protein of NSCLC cells, but promote E-cadherin protein expression, which was in accordance with its effect on cell migration and invasion. To sum up, our data demonstrated that FTH1P3 predicts a poor prognosis and promotes metastasis and aggressiveness in NSCLC by inducing EMT, suggesting FTH1P3 may be a promising target for gene therapy of NSCLC.

Keywords: lncRNA, FTH1P3, NSCLC, features, functions

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide in spite of encouraging advances in diagnosis and treatment [1, 2]. Lung cancer is classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), and NSCLC accounts for about 85% of lung cancer. The majority of NSCLC patients are diagnosed at advanced stages, and the overall five-year survival rate is still as low as 15% due to tumor relapse and distant metastasis [3, 4]. Currently, scientists are committed to study on the development of molecular targets for NSCLC, and some targeted thera-

pies have been revealed to be promising in clinical practice [5]. However, the therapeutic outcomes are still far from optimal. Hence, there is an urgent need to identify the molecular mechanisms of NSCLC progression and to develop innovative therapeutic strategies.

Long non-coding RNAs (lncRNAs) are generally a large group of non-coding RNA transcripts more than 200 nucleotides without evident protein coding functions [6, 7]. Up to now, thousands of lncRNAs have been identified by virtue of chromatin signature analysis and large scale sequencing. These lncRNAs are involved in a wide range of biological processes by regulat-

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ing gene activation and inactivation at transcriptional or post-transcriptional levels [8, 9]. Many studies have demonstrated that the ectopic expression of lncRNAs contributed to tumorigenesis and tumor progression of human malignancies by regulating cell growth, autophagy, metastasis, and invasion [10, 11]. For example, lncRNA HMMR-AS1 promotes proliferation and metastasis of lung adenocarcinoma by regulating the miR-138/sirt6 axis [12]. LncRNA LINCO1354 interacting with hnRNP-D contributes to growth and metastasis in colorectal cancer through activating the Wnt/ β -catenin signaling pathway [13]. LncRNA DLEU1 promotes cell growth, invasion, and confers cisplatin resistance in bladder cancer by regulating the miR-99b/HS3ST3B1 axis [14]. Therefore, the characterization of lncRNAs functions and their clinical significance in NSCLC progression is crucial to develop a possible biomarker and therapy target for NSCLC.

Ferritin heavy chain 1 pseudogene 3 (FTH1P3), a novel tumor-associated lncRNA, is transcribed from a genomic region of human chromosome 2p23.3. FTH1P3 has been identified as an oncogene in oral squamous cell carcinoma [15], uveal melanoma [16], esophageal squamous cell carcinoma [17], and glioma [18]. Although these studies reported the effects of FTH1P3 in different types of human cancer, the biologic functions of FTH1P3 in NSCLC remain poorly understood. Herein, we first focused on FTH1P3 expression patterns in NSCLC tissues and cell lines as compared to that in normal tissues and cell lines. Clinical correlation revealed that high expression of FTH1P3 was associated with advanced TNM stage, lymph node metastasis, and poor prognosis. Finally, cellular functional experiments showed that FTH1P3 promoted NSCLC cell migration and invasion by inducing epithelial-mesenchymal transition (EMT). Collectively, our findings may provide better understanding about the biologic role of FTH1P3 in NSCLC metastasis and aggressiveness.

Material and methods

Patients and tissue collection

The current study was approved by the Ethic Committee of Shenzhen Longhua District Central Hospital. Signed informed consents were

obtained from all patients. From January 2012 to December 2016, a total of 60 pairs of NSCLC tumor tissues and matched normal tissues were collected from patients who underwent lung resection at Shenzhen Longhua District Central Hospital, including 33 males and 27 females. The included patients were aged between 19 to 76 years, with mean age of 48.14 ± 6.87 years. Patients did not receive any radiotherapy or chemotherapy prior to surgery. The tumor tissues and matched normal tissues were confirmed by at least three experienced pathologists. The clinicopathologic features of patients were collected and analyzed. Specimens were rapidly frozen in liquid nitrogen and immediately transferred to a -80°C freezer immediately for subsequent experiments. In this study, the 5-year overall survival time (OS) was defined as the interval between the surgical lung resection date and the end date of follow-up or the date of death owing to recurrence of NSCLC and its associated complications.

Cell lines and cell culture

Human lung epithelial cell line (BEAS-2B), human bronchial epithelial cell line (HBE) and three NSCLC cell lines (A549, H596, and SPCA1) were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The cells were maintained in a humidified incubator at 37°C in a CO_2 humidified atmosphere.

Western blotting

Total protein extracted from NSCLC cells by using lysis buffer supplemented with protein inhibitor and 100 $\mu\text{g}/\text{mL}$ PMSF (Beyotime Biotechnology, Shanghai, China). The same amount of 30 μg protein was separated by 8% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). After blocking with 5% (w/v) skim milk in Tris-buffered saline with Tween-20 (TBST), the membranes were incubated at 4°C overnight with primary antibodies: N-cadherin (#4061S, 1:1000; Cell Signaling Technology, MA, USA), E-cadherin (#14472S, 1:2000; Cell Signaling Technology), vimentin (#5741S, 1:

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1000; Cell Signaling Technology), Snail (#3879S, 1:500; Cell Signaling Technology), and GAPDH (#ab181602, 1:5000; Abcam, CA, USA). After being rinsed with TBST, proteins were then detected by enhanced chemiluminescence system (ECL) reagent (Millipore, MA, USA) after incubation with secondary antibodies for 1 h at room temperature.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The total RNA was extracted from the NSCLC tissues and cells with the help of a RNAeasy™ Animal RNA Isolation Kit with Spin Column (Beyotime Biotechnology, Shanghai, China). To reverse transcribe the cDNA, a PrimeScript™ RT reagent Kit (Takara, Beijing, China) was applied by using 1 µg of the extracted RNA. The cDNA was then used as a template for qRT-PCR assay with the help of a TB Green Premix Ex Taq (Takara, Beijing, China), according to the manufacturer's instructions. The specific primer sequences were synthesized by Sangon Biological Engineering Technology and Service (Shanghai, China). The sequences of primers were as followed: FTH1P3, forward 5'-TCCATTTACCTGTGCGTGGC-3' and reverse 5'-GAAGGAAAGATTCGGCCACCT-3'; GAPDH, forward 5'-GAAGGTGAAGGTCCGGAGTC-3', reverse 5'-GAAGATGGTGATGGGATTTC-3'. GAPDH served as an endogenous control to normalize FTH1P3 expression. The relative expression of FTH1P3 was calculated using the $2^{-\Delta\Delta Cq}$ method [19].

Plasmid construction and cell transfection

Recombinant lentiviral vectors expressing short hairpin RNA (shRNA) targeting FTH1P3 (shRNA-FTH1P3) and negative control shRNA (shRNA-NC) were purchased from Cyagen Biosciences Inc. (Suzhou, China). The shRNA-FTH1P3 and shRNA-NC were cloned into pCMV-VSVG vector to produce recombinant plasmids. The recombinant plasmids were then transfected into 293T cells and generated self-inactivating VSV-G pseudotyped lentivirus. Next, higher purity virus particles were obtained by sucrose ultracentrifugation. For cell transfection, A549 and H596 cells were transfected with shRNA-FTH1P3 and shRNA-NC lentivirus by using Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer's instructions. Subsequently, the cells were collected 48 h

after the transfection and used for further studies.

Wound healing assay

1×10^6 transfected cells were seeded in 6-well plates and grown overnight until reached 100% confluence. Then, three linear wounds in the cellular monolayer were created by scraping the confluent cell monolayer with a 100-µl sterile pipette tip. The suspended cells were washed with PBS twice and grown in DMEM medium without FBS for 24 h. The migration of cells was observed at 0 and 24 h after wounding and then imaged under a phase contrast microscope (Olympus, Tokyo, Japan). The migrating distance of cells was calculated in five random fields by ImageJ software (National Institutes of Health, MD, USA).

Transwell invasion assay

The prepared Matrigel was dissolved in the apical chamber of an incubator. Next, 200 µl DMEM medium free of serum was added into the chamber. After 24-h transfection, the transfected cells were detached by trypsin, centrifuged, and resuspended in the apical chamber to a density of 5×10^4 cells/200 µl. The basolateral chamber was added with 500 µl DMEM medium containing 10% FBS as chemotactic factor. After being cultured for 24 h, the cells that did not move through the Matrigel on the apical chamber were removed using cotton slivers. The invasive cells were fixed with 70% ethanol for 40 min at 37°C, and stained with 0.1% crystal violet (Sigma-Aldrich, MO, USA) for 10 min at 37°C. Stained cells were counted in five randomly selected fields under an IX50 inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as mean \pm standard deviation (SD). All statistical analyses were analyzed by the SPSS 17.0 software (SPSS, Chicago, IL, USA) and from at least three independent experiments. Comparison between two groups was analyzed by Student's t-test. Differences between the groups were analyzed with a one-way analysis of variance (ANOVA) followed by Dunnett's test. Correlations of FTH1P3 expression and clinicopathologic characters of NSCLC patients were evaluated by chi-square test.

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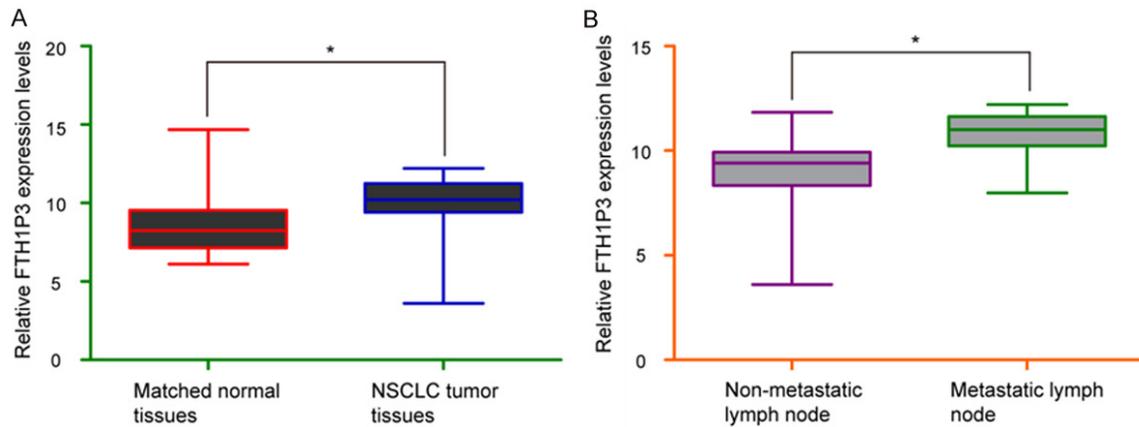


Figure 1. FTH1P3 is upregulated in NSCLC tumor tissues and NSCLC-derived cell lines. A. qRT-PCR analysis of FTH1P3 expression in NSCLC tumor tissues and matched normal tissues from 60 patients. GAPDH was used as an internal control, $n = 3$. B. Metastatic NSCLC tumors had higher FTH1P3 expression levels compared with those of nonmetastatic NSCLC tumors ($n = 3$). * $P < 0.05$ vs control. FTH1P3: ferritin heavy chain 1 pseudogene 3; NSCLC: non-small cell lung carcinoma; qRT-PCR: quantitative real-time PCR.

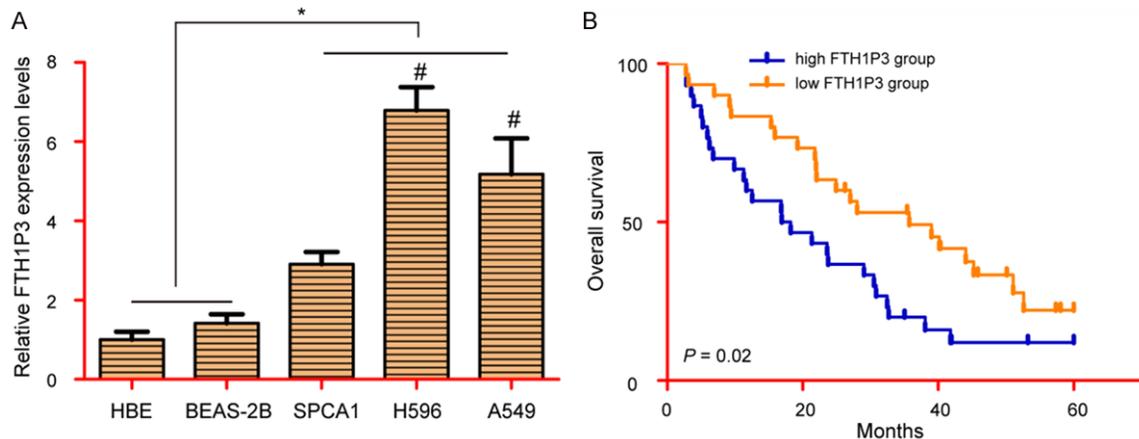


Figure 2. Increased expression of FTH1P3 is associated with poor prognosis in NSCLC patients. A. qRT-PCR analysis of FTH1P3 expression in three NSCLC cell lines (A549, H596, and SPCA1), human lung epithelial cell line (BEAS-2B), and human bronchial epithelial cell line (HBE). B. Kaplan-Meier survival analysis showing correlation between FTH1P3 expression levels and overall survival rate of patients with NSCLC. * $P < 0.05$ vs HBE or BEAS-2B, # $P < 0.05$ vs SPCA1.

Kaplan-Meier method and log-rank test were applied to survival analysis. P value < 0.05 was considered significant.

Results

FTH1P3 is upregulated in NSCLC tumor tissues and NSCLC-derived cell lines

To investigate the relationship between FTH1P3 and NSCLC development, we identified the expression levels of FTH1P3 in 60 pairs of NSCLC tumor tissues and matched normal tissues by qRT-PCR. As shown in **Figure 1A**, the levels of FTH1P3 were significantly upregulated

in NSCLC tumor tissues compared with matched normal tissues ($P < 0.05$). The expression levels of FTH1P3 expression in metastatic lymph node specimens were strikingly elevated in comparison to primary non-metastatic lymph node specimens (**Figure 1B**, $P < 0.05$). Furthermore, expression of FTH1P3 also was detected in NSCLC-derived cell lines. All three NSCLC cell lines had higher FTH1P3 expression than the normal BEAS-2B and HBE cell lines; and of these, A549 and H596 cells had higher FTH1P3 expression than SPCA1 cells (**Figure 2A**, $P < 0.05$), so A549 and H596 cells were selected for subsequent study.

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Table 1. Correlation between clinicopathologic features and FTH1P3 expression levels in 60 NSCLC patients

Variables	Number of cases (n = 60)	Low FTH1P3 expression (n = 30)	High FTH1P3 expression (n = 30)	P
Age (years)				
< 60	38	17	21	0.28
≥ 60	22	13	9	
Sex				
Male	33	15	18	0.44
Female	27	15	12	
Smoking history				
Yes	46	20	26	0.07
No	14	10	4	
Tumor location				
Left lobe	19	11	8	0.41
Right lobe	41	19	22	
Tumor size (cm)				
≥ 3	40	17	23	0.10
< 3	20	13	7	
Histology				
Adenocarcinoma	28	12	16	0.30
Squamous cell carcinoma	32	18	14	
Differentiation				
High	9	3	6	0.28
Middle-low	51	27	24	
TNM stage				
III + IV	23	7	16	0.02*
I + II	37	23	14	
Lymph node metastasis				
Yes	25	8	17	0.02*
No	35	22	13	

FTH1P3, ferritin heavy chain 1 pseudogene 3; NSCLC, non-small cell lung carcinoma.

*Indicates the P values which were less than 0.05.

Increased expression of FTH1P3 is associated with clinical progression and poor prognosis in NSCLC patients

To further explore the potential clinical significances of FTH1P3 in NSCLC, first, chi-square test was performed to detect whether FTH1P3 expression was associated with clinicopathologic findings of NSCLC patients. NSCLC patients were categorized into high FTH1P3 expression group (n = 30) and low FTH1P3 expression group (n = 30) on the basis of the median value of FTH1P3 expression levels. As shown in **Table 1**, high FTH1P3 expression was associated with advanced TNM stage and lymph node metastasis ($P < 0.05$), but no sig-

nificant correlation existed between FTH1P3 expression and other clinicopathologic features, including age, sex, smoking history, tumor size, location, and differentiation, and histology. These results revealed that high FTH1P3 expression was associated malignant progression in NSCLC patients. Next, Kaplan-Meier was performed to assess the prognostic value of FTH1P3 in NSCLC, and found that patients in the low FTH1P3 group achieved OS prolongation in comparison with those in the high FTH1P3 group (**Figure 2B**, $P = 0.02$). Taken together, these findings indicate that increased expression of FTH1P3 correlates with clinical progression and poor prognosis in NSCLC patients.

FTH1P3 promotes NSCLC cell migration and invasion

The above results showed that FTH1P3 expression was increased in metastatic lymph node specimens and associated lymph node metastasis in NSCLC patients, and FTH1P3 has been confirmed to function as a metastasis-associated lncRNA in other human malignancies [16, 17]. Therefore, we supposed that FTH1P3 promotes NSCLC cell migration and invasion *in vitro*. To investigate the biologic roles of FTH1P3 in NSCLC cells, the shRNA-FTH1P3 and shRNA-NC lentiviruses were transfected into A549 and H596 cells, and qRT-PCR was performed to confirm the knockdown efficiency at 48 h post-transfection. The expression of FTH1P3 was markedly decreased after transfection with shRNA-FTH1P3 lentivirus compared with shRNA-NC lentivirus in NSCLC cells (**Figure 3A**, $P < 0.05$). Subsequently, we used a wound healing assay to explore the effect of FTH1P3 on NSCLC cell migration. Images of A549 and H596 cells transfected with shRNA-FTH1P3 and shRNA-NC lentiviruses at 0 h and 24 h after wounding

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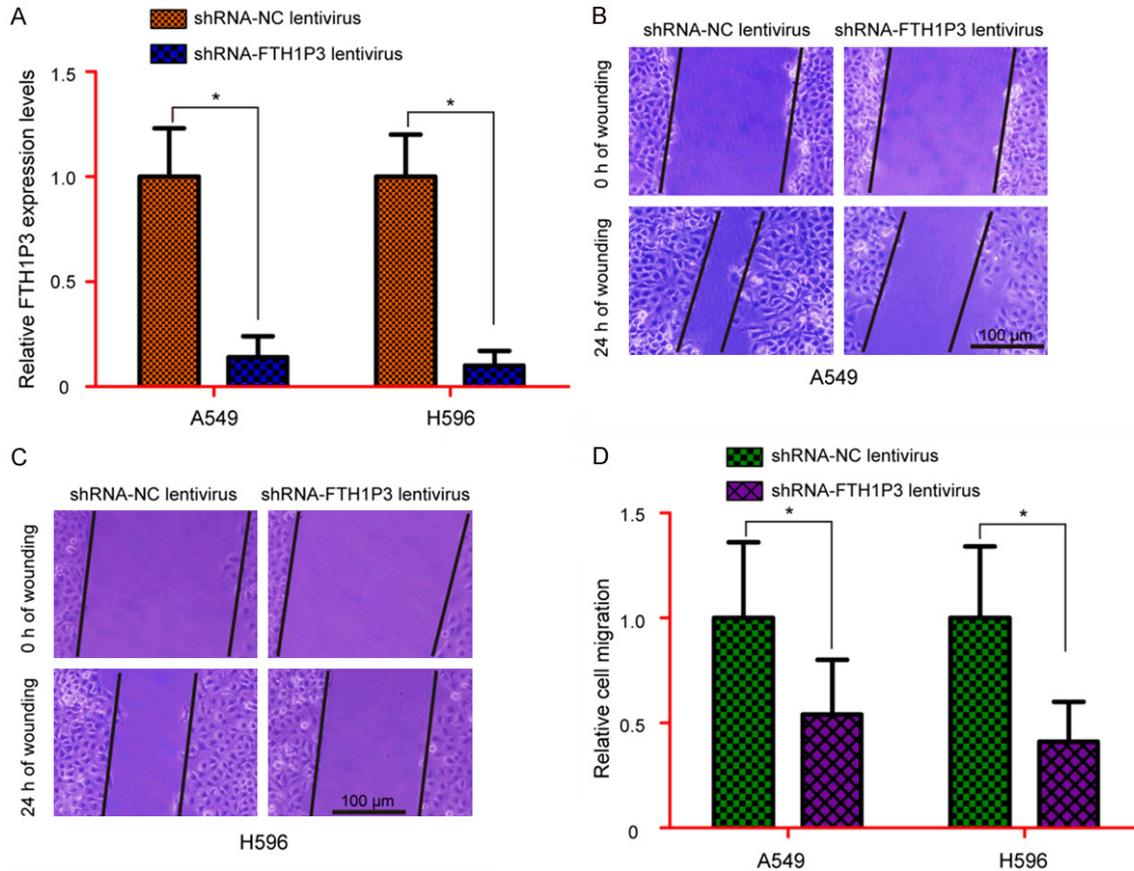


Figure 3. Knockdown of FTH1P3 inhibits cell migration of NSCLC cells. A. The expression levels of FTH1P3 were examined in A549 and H596 cells transfected with the shRNA-FTH1P3 and shRNA-NC lentiviruses ($n = 3$). B. Wound healing assay was used to evaluate the effect of FTH1P3 knockdown on NSCLC cell migration ($n = 3$). The migration distances of A549 cells were observed at 0 and 24 h after wounding. C. The migration distances of H596 cells were observed at 0 and 24 h after wounding. D. Knockdown of FTH1P3 strongly inhibited migratory capability ($n = 3$). ShRNA: short hairpin RNA; shRNA-NC: negative control shRNA. * $P < 0.05$ vs shRNA-NC lentivirus.

are shown in **Figure 3B** and **3C**. The results showed that knockdown of FTH1P3 strongly inhibited the migratory capability (**Figure 3D**, $P < 0.05$). Moreover, a transwell invasion assay was performed to investigate the role of FTH1P3 on the invasion of NSCLC cells. Cell invasion of A549 and H596 cells are presented in **Figure 4A** and **4B**. The data show that downregulation of FTH1P3 significantly suppressed the invasion ability of A549 and H596 cells compared with the shRNA-NC lentivirus groups ($P < 0.05$). In sum, these results suggest that FTH1P3 promotes NSCLC cell migration and invasion.

FTH1P3 facilitates NSCLC cell migration and invasion by inducing EMT

Since EMT is closely correlated with tumor metastatic abilities, we then examined several EMT

markers in A549 and H596 cells transfected with shRNA-FTH1P3 and shRNA-NC lentiviruses. Consistent with our hypothetical conclusion, western blotting results showed that the expression of E-cadherin was higher in shRNA-FTH1P3 lentivirus-transfected groups compared with that in shRNA-NC lentivirus groups, whereas the levels of N-cadherin, vimentin and Snail were obviously downregulated in A549 and H596 cells transfected with shRNA-FTH1P3 lentivirus (**Figure 5A** and **5B**, $P < 0.05$). Downregulation of the EMT transcription factor Snail and activation of the epithelial marker E-cadherin along with the downregulation of mesenchymal markers N-cadherin and vimentin demonstrated the inhibition of EMT. Together, these results indicated that FTH1P3 facilitates metastasis and aggressiveness in NSCLC by inducing EMT.

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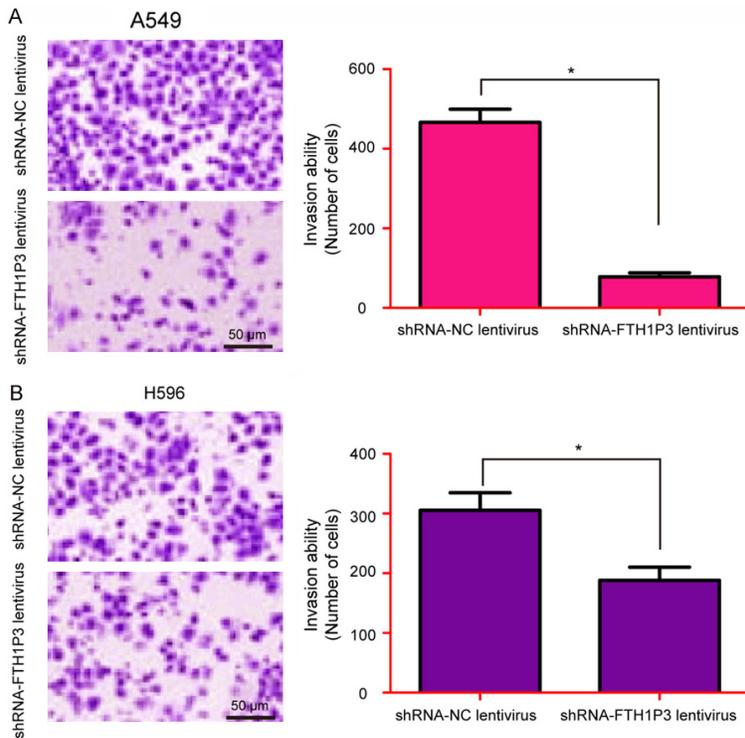


Figure 4. FTH1P3 inhibition suppresses invasion of NSCLC cells. A. Transwell invasion assay was used to evaluate the effect of FTH1P3 knockdown on NSCLC cell invasive capacity. Markedly reduced cell invasion in A549 cells following knockdown of FTH1P3 by shRNA-FTH1P3 ($n = 3$). B. Representative images of shRNA-FTH1P3 and shRNA-NC lentiviruses transfected H596 cells. Downregulation of FTH1P3 significantly suppressed the invasion ability of H596 cells ($n = 3$). * $P < 0.05$ vs shRNA-NC lentivirus.

Discussion

lncRNAs have been reported to be involved in the biological progression of NSCLC, and aberrant expression of lncRNAs is closely associated with tumor growth, metastasis and angiogenesis, thus providing a potential target for the diagnosis and therapy of NSCLC [20, 21]. Even though several studies reported the dysregulation of FTH1P3 expression in oral squamous cell carcinoma [15], uveal melanoma [16], esophageal squamous cell carcinoma [17] and glioma [18], its biologic functions and clinical implications in NSCLC remained unclear. In this study, we performed qRT-PCR assay for analysis of the expression of FTH1P3 and found that FTH1P3 expression was significantly upregulated in NSCLC tumors tissues and cell lines compared with matched normal tissues and normal cells. In addition, the expression levels of FTH1P3 expression in metastatic lymph node specimens were strikingly elevated

in comparison to primary non-metastatic lymph node specimens. Interestingly, based on the statistical analysis, increased expression of FTH1P3 correlates with advanced TNM stage, lymph node metastasis, and poor prognosis in NSCLC patients, suggesting that FTH1P3 may play an universal and important role during tumor progression and metastasis of NSCLC.

FTH1P3, located in human chromosome 2p23.3, has been associated with the development of numerous human cancer types. Zhang et al. [15] demonstrated that FTH1P3 is overexpressed in oral squamous cell carcinoma (OSCC) and decreases the survival rate of OSCC patients. Ectopic expression of FTH1P3 facilitates cell proliferation and colony formation in OSCC cells. Zheng et al. [16] reported that FTH1P3 is upregulated in uveal melanoma cell lines and tissues; elevated expression of FTH1P3 promoted uveal melanoma cell proliferation, cell cycle, and migration.

In addition, Wang et al. [22] confirmed the potential regulatory mechanism of FTH1P3 on breast cancer paclitaxel resistance through miR-206/ABCB1 and provided a novel insight for the breast cancer chemoresistance. Notably, upregulation of FTH1P3 may have promoted glioma cell proliferation and inhibited apoptosis, and the miR-224-5p/TPD52 axis may be a downstream mechanism of FTH1P3 in glioma progression [18]. To the best of our knowledge, this is the first report to characterize the biologic roles of FTH1P3 in NSCLC. The cytologic tests demonstrated that knockdown of FTH1P3 remarkably inhibited the migration and invasion abilities of NSCLC cells *in vitro*. Our observation was consistent with the findings by Yang et al. [17] who reported that FTH1P3 plays a crucial role in esophageal squamous cell carcinoma (ESCC) metastasis and invasion, and can be used as a potential therapeutic target for ESCC.

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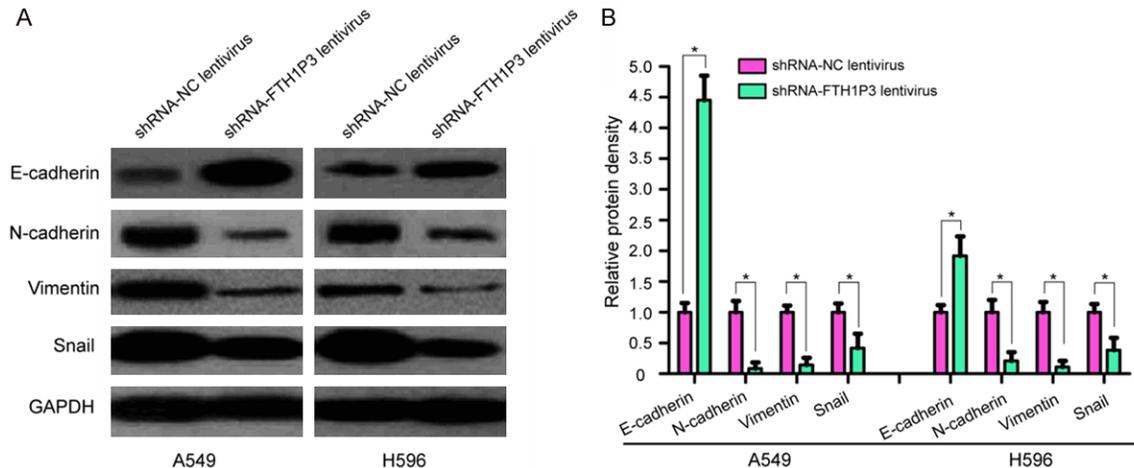


Figure 5. FTH1P3 facilitates NSCLC cell migration and invasion by inducing EMT. A. A549 and H596 cells were transfected with shRNA-FTH1P3 and shRNA-NC lentiviruses, and western blotting was conducted to analyze the expression of E-cadherin, N-cadherin, Snail and vimentin. B. FTH1P3 knockdown could decrease expression of N-cadherin, vimentin and Snail protein of NSCLC cells, but promote E-cadherin protein expression (n = 3). *P < 0.05 vs shRNA-NC lentivirus.

EMT programming participates in tissue repair, embryogenesis, and numerous pathologic conditions, particularly tumor progression and metastasis [23, 24]. EMT is often associated with primary cancer cell dissemination, which leads to metastasis. Phenotypic changes associated with EMT are characterized by the loss of the cell-cell contact, cell polarity, and E-cadherin, which co-occur with an increase in mesenchymal markers such as vimentin and N-cadherin. Since several lncRNAs directly affect EMT-related master regulators, they have the potential to be used as biomarkers or targets in EMT-based pathologic conditions such as NSCLC [25, 26]. Therefore, comprehensive understanding of lncRNAs-EMT correlation with tumor metastatic spread may provide improvements to diagnostic tools or therapeutics for NSCLC. In this study, we examined key biomarkers of EMT, such as E-cadherin, N-cadherin, Snail and vimentin, and found that FTH1P3 knockdown could decrease expression of N-cadherin, vimentin, and Snail protein of NSCLC cells, but promote E-cadherin protein expression. Therefore, the above mentioned findings indicated that FTH1P3 could promote EMT, which was in accordance with its promoting role on migration and invasion of NSCLC cells.

Taken together, our findings revealed that FTH1P3 is upregulated in NSCLC tumor tissues and NSCLC-derived cell lines, and its expression has correlation with clinical progression

and poor prognosis. FTH1P3 facilitates the metastasis and aggressiveness in NSCLC by inducing EMT, suggesting FTH1P3 as a promising target for gene therapy of NSCLC.

Disclosure of conflict of interest

None.

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