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ORIGINAL ARTICLE

Interferon induced transmembrane protein 3 regulates the growth and invasion of human lung adenocarcinoma

Dong Zhang[1,2], Huimin Wang[2], Huijie He[2], Haiying Niu[2] & Yu Li[1]

1 Department of Pulmonary Medicine, Qilu Hospital, Shandong University, Jinan, China
2 Department of Pulmonary Medicine, The First Affiliated Hospital of Baotou Medical College, Baotou, China

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Correspondence
Yu Li, Department of Pulmonary Medicine, Qilu Hospital, Shandong University, 107 West Wenhua Rd, Jinan, Shandong 250012, China.
Tel: +86 531 8216 9330
Fax: +86 531 8692 7544
Email: qiluliyu@163.com

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Abstract

Background: Interferon induced transmembrane protein 3 (IFITM3) plays an important role in the tumorigenesis and progression of multiple cancers. This study investigated the expression and function of IFITM3 in human lung adenocarcinoma.

Methods: Fifty human lung adenocarcinoma tissues were collected. IFITM3 expression was assessed by immunohistochemical staining. The clinicopathologic characteristics of all patients were analyzed.

Results: IFITM3 was mainly detected in the cytoplasm of advanced cancer tissues and its expression was correlated with tumor malignancy grade. Knockdown of IFITM3 in vitro markedly inhibited the proliferation and invasion of lung adenocarcinoma cells.

Conclusion: IFITM3 represents a potential therapeutic target for the treatment of lung adenocarcinoma.

Introduction

Cancer is the most frequent cause of death worldwide.[1] Lung cancer is the leading cause of cancer death, as a result of the uncontrolled proliferation of cells in lung tissue.[2] Both genetic and environmental factors are involved in the etiologies of lung cancer.[3,4] Long-term exposure to tobacco smoke, genetic factors, and air pollution are the main causes of lung cancer.[5–7] Numerous studies have shown that lung cancer develops as a result of genetic alterations that accumulate with tumor progression and it therefore shows great morphological and genetic heterogeneity in its molecular profile and response to therapy.[8–12] Many genes and proteins have been identified as crucial to the carcinogenesis of lung cancer; however, the exact mechanisms of lung cancer are still poorly understood.[13]

Interferon induced transmembrane protein 3 (IFITM3, also known as 1-8U) was first identified as an antiviral effector in complementary DNA screening from interferon-treated T98G neuroblastoma cells.[14] IFITM3 messenger (m)RNA is detected in most tissues and its expression level is highly inducible by interferon.[15] Further analysis showed that IFITM3 belongs to the interferon-inducible transmembrane protein (IFITM) family composed of five IFITM members, including IFITM1, 2, 3, 5, and 10 in humans.[16–18] All members of this protein family have two putative transmembrane domains interspersed by a conserved cytoplasmic region.

Recent studies have demonstrated that members of IFITM family may be crucial factors in carcinogenesis. IFITM1 and IFITM3 expression levels were reportedly much higher in astrocytoma cells than in normal astrocytes.[19] Furthermore, upregulation of IFITM1 affected the proliferation and invasion of glioma.[20] IFITM2 also played a critical role in regulating cell death through a p53-independent proapoptotic signaling pathway.[21] IFITM3 was found at higher levels in colon tumors than in normal colon tissue, indicating a potential role of IFITM3 in carcinogenesis.[22] However, the precise role of IFITM3 in lung cancer is still unknown.

In this study we first detected the expression of IFITM3 in lung cancer tissues. To investigate the potential role of

IFITM3 in lung cancer, we employed a loss of function approach using IFITM3-specific short hairpin RNA (Lv-shIFITM3) to knockdown IFITM3 in lung cancer cell lines. The proliferation, colony formation, and cell cycle of lung cancer cells were examined. Our results showed that IFITM3 is a critical factor regulating carcinogenesis of lung cancer and may be a promising target for treatment.

Methods

Materials

Human lung cancer cell lines A549 and H1299 were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). Lv-shIFITM3 and negative control vectors were obtained from Genechem (Shanghai, China). All antibodies (anti-IFITM3, 1:50 dilution; SAB1410086) and chemicals used were obtained from Sigma (St. Louis, MO, USA).

Immunohistochemical staining

Fifty lung adenocarcinoma (LAC) tissues were surgically obtained between June 2010 and December 2015. Tissues were fixed in 10% formalin solution and embedded in paraffin. Histological slices of 3 mm were prepared, deparaffined in xylene, and rehydrated with ethanol. Endogenous peroxidase was inhibited by incubation with freshly prepared 3% hydrogen peroxide with 0.1% sodium azide. The sections were incubated with IFITM3 antibody overnight at 4°C, and then incubated with secondary anti-mouse antibody for 30 minutes at room temperature. After washing, the slides were treated with diaminobenzidine solution and counterstained with hematoxylin. Phosphate buffered saline replaced anti-IFITM3 in negative controls.

Quantitative real-time PCR

Total RNA was extracted from A549 and H1299 cells with TRIzol according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Reverse-transcription was carried out using Moloney Murine Leukemia Virus (M-MLV, Sigma) while complementary DNA amplification was carried out using a SYBR Green Master Mix kit (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: IFITM3, 5′-CGAAACTACTGGGGAAAGGGA-3′ and 5′- ATTCATGGTGTCCAGCGAAGA -3′; GAPDH, 5′-CAACGAATTTGGCTACAGCA-3′ and 5′-AGGGGTCTACATGGCAACTG-3′. Data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) in triplicate.

Cell culture and transfection

A549 and H1299 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Lv-shIFITM3 and negative control lentivirus were used to infect A549 and H1299 cells. Cells were subcultured at a 1:5 dilution in medium containing 300 μg/mL G418. Positive stable transfectants were selected and expanded for further analysis.

Cell proliferation assay

Cell proliferation was analyzed with methyl-thiazolyl-tetrazolium (MTT) assay. Briefly, cells were trypsinized, resuspended, and seeded into 96-well plates at a density of 1×10^5 cells/well. Cells were treated with 20 μL MTT and then incubated with 150 μL of dimethyl sulfoxide (DMSO) for five minutes. The color reaction was measured at 570 nm with an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA).

Transwell invasion and migration assay

Transwell filters were coated with matrigel (Costar, Cambridge, MA, USA) on the upper surface of a polycarbonic membrane. To analyze cell invasion, 1×10^5 cells in 100 μL serum-free DMEM were added into the upper compartment of the chamber, while 200 μL conditioned medium was placed in the bottom compartment of the chamber as chemoattractant. After incubation for 24 hours, the cells on the upper surface of the membrane filter were removed, and the cells that had penetrated to the lower surface of the membrane were fixed and stained with crystal violet. Migration assays were performed using a Transwell compartment (Costar) without matrigel. Each assay was repeated three times.

Cell cycle assay

Cells were seeded in six-well plates at 2×10^5 cells/well and cultured for 24 hours. After adhesion, the cells were harvested and centrifuged at 1500 rpm for five minutes. Pellets were washed with phosphate buffered saline, fixed with ethanol at 4°C for 30 minutes, and stored at −20°C overnight. The cells were collected after centrifugation at 1500 rpm for five minutes and incubated with propidium iodide containing RNase A at 4°C for 30 minutes. Cell suspension was filtered through a mesh filter and analyzed by flow cytometry.

Statistical analysis

SPSS version 13.0 (SSPS Inc., Chicago, IL, USA) was used for statistical analysis. Kruskal–Wallis, chi-square and *t*-tests were used to compare the groups. A Wilcoxon rank sum test was used to analyze the correlation between protein expression and clinicopathologic features. Significance was defined as $P < 0.05$.

Results

Interferon induced transmembrane protein 3 (IFITM3) protein expression in human lung adenocarcinoma

Interferon induced transmembrane protein 3 expression levels in 50 LAC tissues were evaluated using immunohistochemical staining. The cases were divided into two groups according to pathologic tumor node metastasis staging: stage I and II tumors with the least malignant phenotypes, and stage III and IV tumors with the most malignant phenotypes. IFITM3 was positively stained in the cytoplasm of advanced cancer tissues, but was negatively or weakly stained in early stage cancer specimens (Fig 1). IFITM3 expression levels were associated with tumor differentiation, lymph node, distant metastasis, and tumor node metastasis stages (Table 1).

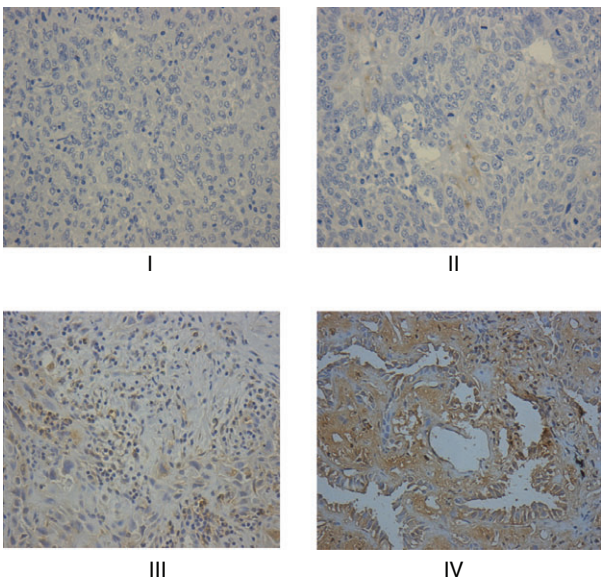

Figure 1 Interferon induced transmembrane protein 3 (IFITM3) expression in human lung adenocarcinoma (LAC) was examined by immunohistochemistry (stages I–II and III–IV). In human LAC, IFITM3 protein was mainly stained in the cytoplasm of advanced cancer tissues and its expression levels increased with ascending tumor malignancy grade.

Table 1 Correlation of IFITM3 overexpression with clinicopathologic characteristics of LAC

Variables	Cases (*n*)	IFITM3 overexpression		*P*
		Score < 2	Score ≥ 2	
Total	50	22	28	
Age (year)				>0.05
<60	29	13	16	
≥60	21	9	12	
Gender				>0.05
Male	27	12	15	
Female	23	10	13	
TNM staging				<0.05
I + II	26	14	12	
III + IV	24	8	16	
Tumor size				>0.05
T1–T2	32	15	17	
T3–T4	18	7	11	
Lymph node metastases				<0.05
No	29	15	14	
Yes	21	7	14	

IFITM3, interferon induced transmembrane protein 3; LAC, lung adenocarcinoma; TNM, tumor node metastasis.

Knockdown of IFITM3 in H1299 cells by Lv-shIFITM3

To examine the role of IFITM3 in LAC progression, mRNA expression levels of IFITM3 in A549 and H1299 cells were detected by real-time PCR. As shown in Figure 2a, IFITM3 expression was higher in H1299 than in A549 cells. Therefore, H1299 cells were selected for further analysis. Lv-shIFITM3 was transfected into H1299 cells, and we observed obvious inhibition of IFITM3 mRNA expression in the Lv-shIFITM3 group compared to the normal control (NC) group (Fig 2b).

To detect the transfection efficiency of Lv-shIFITM3, H1299 cells infected with Lv-shIFITM3 and Lv-shCon were observed under fluorescence microscope. More than 90% of the cells expressed green fluorescent protein at 96 hours after infection, which indicated high efficiency infection by Lv-shIFITM3 (Fig 2c).

IFITM3 knockdown inhibited adenocarcinoma cell proliferation

Abnormal cell proliferation is a hallmark of cancer. First, we investigated the proliferative activities of H1299 cells by MTT. We found that IFITM3 knockdown could significantly suppress the proliferative activities of H1299 cells in a time-dependent manner compared to the NC group (Fig 3).

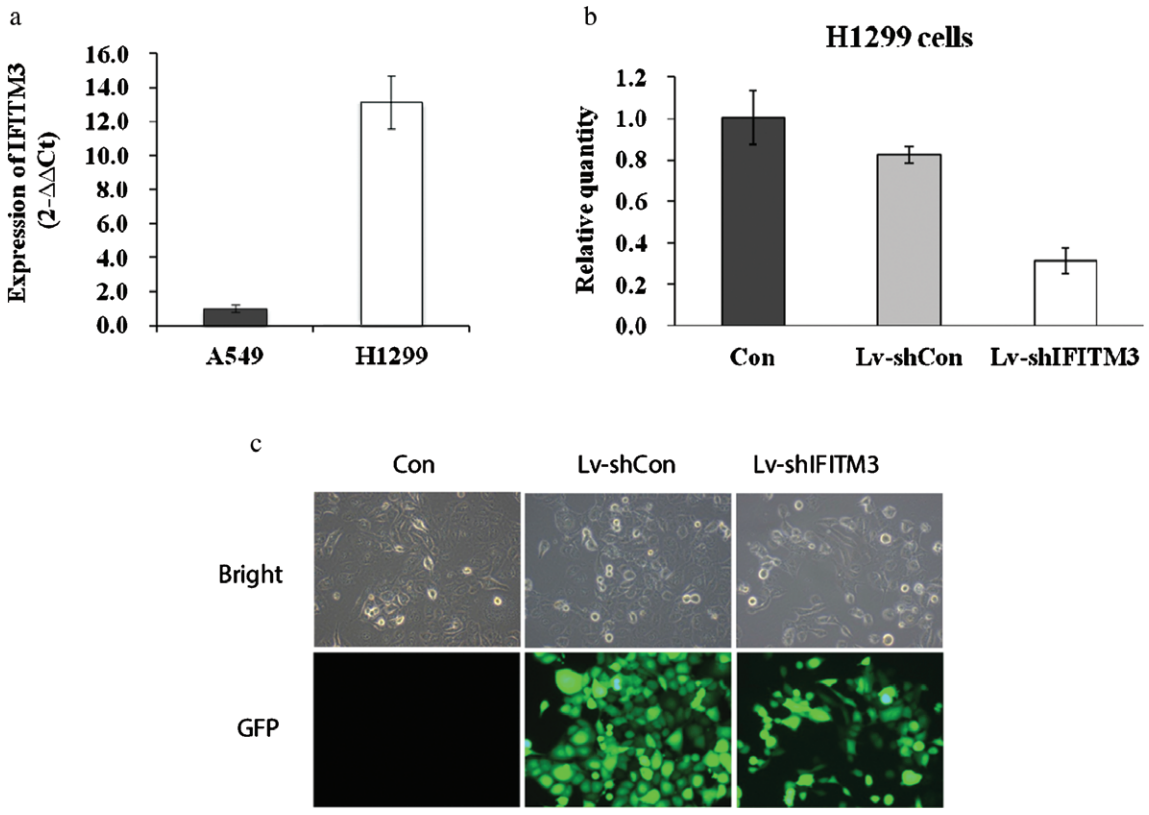

Figure 2 Short hairpin RNA mediated knockdown of interferon induced transmembrane protein 3 (IFITM3). The messenger (m)RNA expression level of IFITM3 was measured by (**a**) real-time PCR in A549 and H1299 cells and (**b**) real-time PCR in H1299 cells infected by Lv-shIFITM3 or Lv-shControl. (**c**) Representative images of H1299 cells after three days of lentivirus infection. Infected cells expressed green fluorescent protein.

IFITM3 knockdown inhibited adenocarcinoma cell invasion and migration

Transwell assay was carried out to determine the effects of IFITM3 knockdown on the invasive and metastatic potential of H1299 cells. The results indicated that the invasion and migration potential of H1299 cells distinctly decreased after IFITM3 knockdown. Representative micrographs of the Transwell assay are shown in Figure 4a,b. Quantitative analysis showed that the invasive activities of H1299 cells decreased significantly in the Lv-shIFITM3 group compared to the NC group (**$P < 0.01$) (Fig 4c).

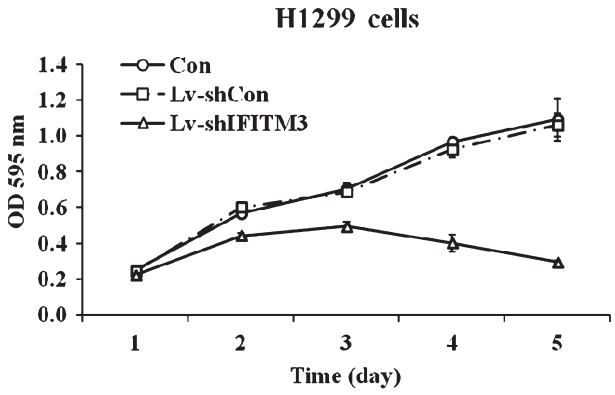

Figure 3 Interferon induced transmembrane protein 3 (IFITM3) knockdown inhibited lung adenocarcinoma cell proliferation. Methyl-thiazolyl-tetrazolium assay showed that IFITM3 knockdown could significantly suppress the proliferative activities of H1299 cells in a time-dependent manner compared to the control.

IFITM3 knockdown induced adenocarcinoma cell cycle arrest and apoptosis

Flow cytometry analysis showed that compared to the NC group, there was a significant increase in the cell population at the G0/G1 phase, and a decrease in the cell population at the G2/M and S phases following IFITM3 knockdown (Fig 5). These results indicated that IFITM3 knockdown arrests H1299 cells in the G0/G1 phase.

Discussion

Lung cancer is the leading cause of cancer death worldwide, with an estimated 15% overall five-year survival rate.

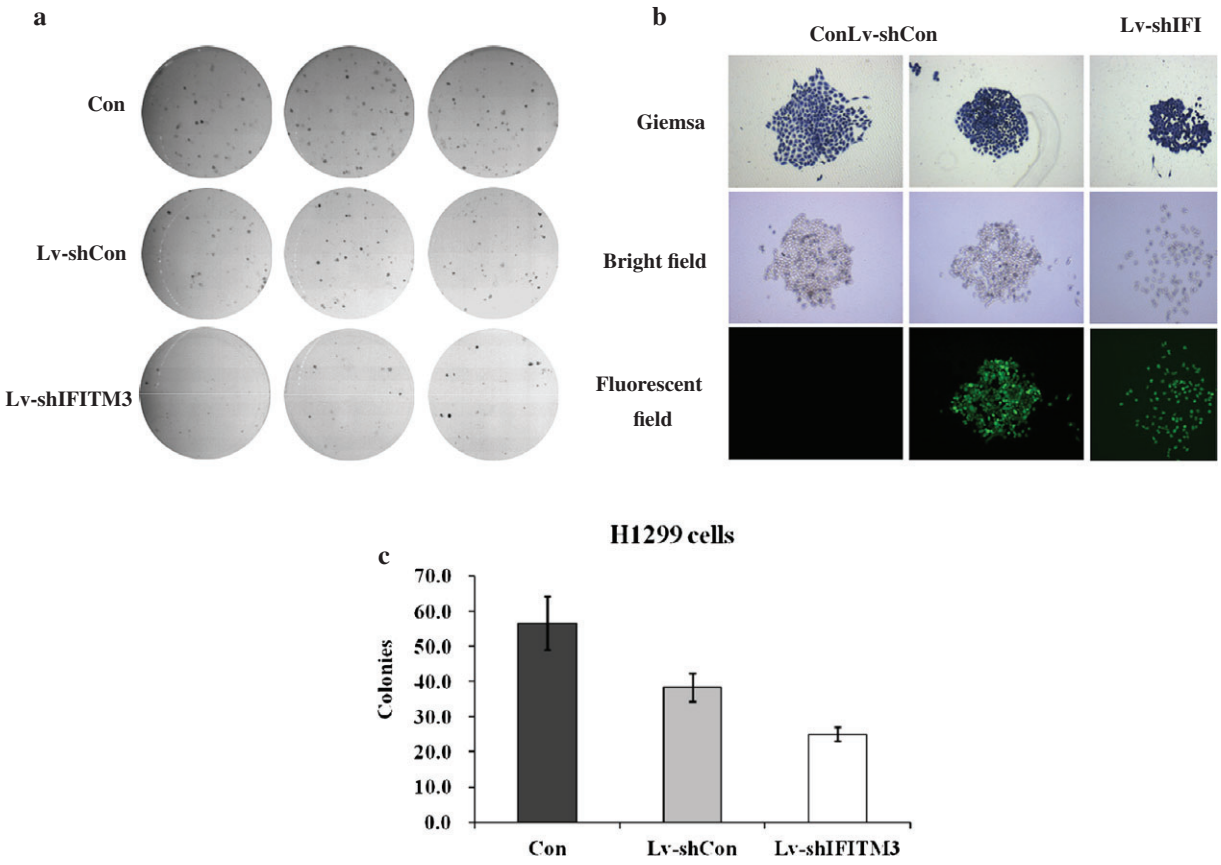

Figure 4 Interferon induced transmembrane protein 3 (IFITM3) knockdown inhibited lung adenocarcinoma cell invasion and migration. Representative micrographs of (**a**) Transwell invasion assay and (**b**) Transwell migration assay. (**c**) Quantitative analysis of the number of invaded colonies with Giemsa staining.

Thus, it is very important to understand the molecular signaling pathways and identify the key modulators that induce lung cancer progression.

Lentivirus-mediated gene silencing technology has been demonstrated as a powerful tool to knockdown specific genes in vivo and in vitro through a posttranscriptional mechanism.[23–25] In this study, lentivirus-mediated gene silencing technology was employed to inhibit the expression of endogenous IFITM3 in H1299 lung cancer cells. Cells with low IFITM3 expression levels demonstrated impaired proliferation, colony formation, and cell cycle regulation. In addition, we found higher IFITM3 expression in the majority of human lung cancer tissues compared to adjacent NC tissues. These results suggest that IFITM3 may be a critical oncogene in the carcinogenesis of lung adenocarcinoma.

However, some studies have suggested that IFITM3 may act as a tumor suppressor. It has been reported that IFITM3 could interact with osteopontin (OPN), a protein that plays an important role in tumor progression, both in vitro and in vivo.[26] Further studies showed that IFITM3 reduced the mRNA expression level of OPN by affecting OPN mRNA stability. Another study reported that IFTTM3 induced by interferon-alpha could suppress the proliferation of human melanoma cells through promoter demethylation of the IFITM3 gene, suggesting an anti-proliferative property of IFITM3.[27,28] In our study, we showed that knockdown of IFITM3 by lentivirus-mediated gene silencing technology suppressed proliferation and induced cell-cycle arrest in lung cancer cells. Furthermore, the majority of patients with lung cancer had elevated IFITM3 expression levels compared to adjacent normal tissue, as illustrated by immunohistochemistry. Our data support the theory that IFITM3 may act as an oncogene in lung adenocarcinoma. The different roles of IFITM3 may partially result from the different signaling pathways that mediate the downstream effects of IFITM3 in different cancer types. However, the exact role of IFITM3 in tumor progression still needs to be explored.

In conclusion, we have demonstrated, for the first time, that IFITM3 is expressed at a much higher level in lung cancer than in control tissues. Furthermore, knockdown of

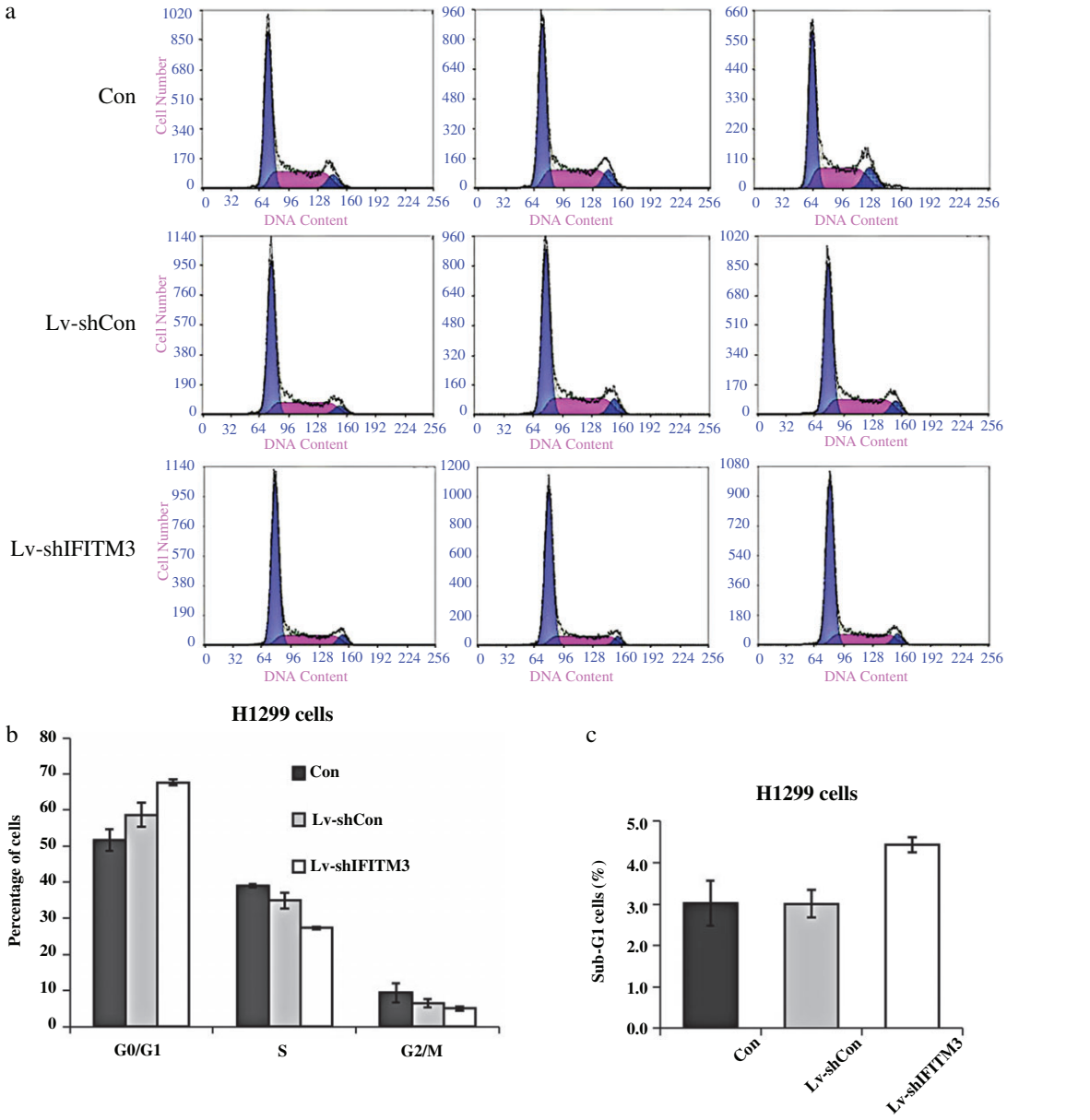

Figure 5 Interferon induced transmembrane protein 3 (IFITM3) knockdown induced lung adenocarcinoma cell cycle arrest and apoptosis. (**a**) Cell cycle distribution of H1299 cells was detected by flow cytometry. (**b**) Compared to the control, the population of cells in the G0/G1 phase increased, and cells in the G2/M and S phases decreased. (**c**) The percentage of apoptotic cells was significantly increased in the Lv-shIFITM3 group.

IFITM3 could suppress lung cancer cell proliferation, invasion, and migration while inducing lung cancer cell cycle arrest and apoptosis. IFITM3 represents a promising therapeutic target for the treatment of lung cancer.

Disclosure

No authors report any conflict of interest.

References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. (Published erratum appears in *CA Cancer J Clin* 2011; 61: 134.) *CA Cancer J Clin* 2011; **61**: 69–90.

2 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893–917.

3 Sos ML, Thomas RK. Genetic insight and therapeutic targets in squamous-cell lung cancer. *Oncogene* 2012; **31**: 4811–4.
4 Sankpal UT, Pius H, Khan M *et al.* Environmental factors in causing human cancers: Emphasis on tumorigenesis. *Tumour Biol* 2012; **33**: 1265–74.
5 Steliga MA, Dresler CM. Epidemiology of lung cancer: Smoking, secondhand smoke, and genetics. *Surg Oncol Clin N Am* 2011; **20**: 605–18.
6 Heist RS, Sequist LV, Engelman JA. Genetic changes in squamous cell lung cancer: A review. *J Thorac Oncol* 2012; **7**: 924–33.
7 Caputi M, Esposito V, Mancini A, Giordano A. Air pollution and respiratory pathology: Lung cancer. *Monaldi Arch Chest Dis* 2002; **57**: 177–9.
8 Yokota J, Shiraishi K, Kohno T. Genetic basis for susceptibility to lung cancer: Recent progress and future directions. *Adv Cancer Res* 2010; **109**: 51–72.
9 Li T, Kung HJ, Mack PC, Gandara DR. Genotyping and genomic profiling of non-small-cell lung cancer: Implications for current and future therapies. *J Clin Oncol* 2013; **31**: 1039–49.
10 Zheng YW, Li RM, Zhang XW, Ren XB. Current adoptive immunotherapy in non-small cell lung cancer and potential influence of therapy outcome. *Cancer Invest* 2013; **31**: 197–205.
11 Massarelli E, Papadimitrakopoulou VA. Phosphatidykinosital 3-kinase and mammalian target of rapamycin pathway in non-small-cell lung cancer. *J Thorac Oncol* 2012; **7** (16 Suppl. 5): S379–82.
12 Ekman S, Wynes MW, Hirsch FR. The mTOR pathway in lung cancer and implications for therapy and biomarker analysis. *J Thorac Oncol* 2012; **7**: 947–53.
13 Helmig S, Schneider J. Oncogene and tumor-suppressor gene products as serum biomarkers in occupational-derived lung cancer. *Expert Rev Mol Diagn* 2007; **7**: 555–68.
14 Friedman RL, Manly SP, McMahon M, Kerr IM, Stark GR. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. *Cell* 1984; **38**: 745–55.
15 Siegrist F, Ebeling M, Certa U. The small interferon-induced transmembrane genes and proteins. *J Interferon Cytokine Res* 2011; **31**: 183–97.
16 Evans SS, Collea RP, Leasure JA, Lee DB. IFN-alpha induces homotypic adhesion and Leu-13 expression in human B lymphoid cells. *J Immunol* 1993; **150**: 736–47.
17 Lange UC, Saitou M, Western PS, Barton SC, Surani MA. The fragilis interferon-inducible gene family of transmembrane proteins is associated with germ cell specification in mice. *BMC Dev Biol* 2003; **3**: 1.
18 Smith RA, Young J, Weis JJ, Weis JH. Expression of the mouse fragilis gene products in immune cells and association with receptor signaling complexes. *Genes Immun* 2006; **7**: 113–21.
19 Seyfried NT, Huysentruyt LC, III Atwood JA, Xia Q, Seyfried TN, Orlando R. Up-regulation of NG2 proteoglycan and interferon-induced transmembrane proteins 1 and 3 in mouse astrocytoma: A membrane proteomics approach. *Cancer Lett* 2008; **263**: 243–52.
20 Yu F, Ng SS, Chow BK *et al.* Knockdown of interferon-induced transmembrane protein 1 (IFITM1) inhibits proliferation, migration, and invasion of glioma cells. *J Neurooncol* 2011; **103**: 187–95.
21 Daniel-Carmi V, Makovitzki-Avraham E, Reuven EM *et al.* The human 1-8D gene (IFITM2) is a novel p53 independent pro-apoptotic gene. *Int J Cancer* 2009; **125**: 2810–9.
22 Li D, Peng Z, Tang H *et al.* KLF4-mediated negative regulation of IFITM3 expression plays a critical role in colon cancer pathogenesis. *Clin Cancer Res* 2011; **17**: 3558–68.
23 Dror S, Sander L, Schwartz H *et al.* Melanoma miRNA trafficking controls tumour primary niche formation. *Nat Cell Biol* 2016; **18**: 1006–17.
24 Stevenson M. Molecular biology of lentivirus-mediated gene transfer. *Curr Top Microbiol Immunol* 2002; **261**: 1–30.
25 Neschadim A, McCart JA, Keating A, Medin JA. A roadmap to safe, efficient, and stable lentivirus-mediated gene therapy with hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2007; **13**: 1407–16.
26 El-Tanani MK, Jin D, Campbell FC, Johnston PG. Interferon-induced transmembrane 3 binds osteopontin in vitro: Expressed in vivo IFITM3 reduced OPN expression. *Oncogene* 2010; **29**: 752–62.
27 Brem R, Oraszlan-Szovik K, Foser S, Bohrmann B, Certa U. Inhibition of proliferation by 1-8U in interferon-alpha-responsive and non-responsive cell lines. *Cell Mol Life Sci* 2003; **60**: 1235–48.
28 Scott R, Siegrist F, Foser S, Certa U. Interferon-alpha induces reversible DNA demethylation of the interferon-induced transmembrane protein-3 core promoter in human melanoma cells. *J Interferon Cytokine Res* 2011; **31**: 601–8.

