ORIGINAL ARTICLE

Activating transcription factor 3 promotes malignance of lung cancer cells *in vitro*

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Abstract

Background: Lung cancer remains the most common cause of cancer-related death, with high rates of recurrence and poor outcomes. An abnormally high expression of activating transcription factor 3 (ATF3) in various cancers suggests an oncogenic role; however, its function in lung cancer is largely unknown.

Methods: Sixty-four pairs of lung cancer tissues were collected for ATF3 expression analysis by quantitative real-time PCR, immunoblotting, and immunohistochemistry staining. Correlations between ATF3 expression with clinicopathological features and overall survival were analyzed. ATF3 expression in a panel of lung cancer cell lines together with normal bronchial epithelial Beas-2B cells was also determined. Human H1299 and A549 cells were used for ATF3 knockdown and/or overexpression assays. Alterations in cell proliferation, cell cycle attribution, migration, and invasion were all assessed *in vitro*.

Results: Increased ATF3 messenger RNA and protein expression were observed in lung cancer tissues/cells compared with normal tissues/cells. High tumorous ATF3 expression was significantly correlated with positive advanced tumor grade, lymph node metastasis, and shorter overall survival. Experimentally, we found that RNA interference mediated knockdown of ATF3 significantly inhibited the cell proliferation, cell cycle progression, migration, and invasion capacities of lung cancer cells *in vitro*, whereas forced expression of ATF3 did the opposite.

Conclusion: Upregulation of ATF3 in lung cancer promotes cell proliferation, migration, and invasion, and may represent a novel therapeutic target for lung cancer.

Introduction

Lung cancer is one of the leading causes of cancer-related mortality in the world, with high incidence and a 16.6% five-year survival rate.[1–3] In China, lung cancer is one of the most common newly diagnosed cancers and the leading cause of cancer death.[4,5] According to histological characteristics, lung cancer can be classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

NSCLC, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, constitutes approximately 85% of all lung cancers, and more than half of the newly diagnosed NSCLC patients have metastatic disease.[6] Despite the progress in treatment modalities, the prognosis actuality of lung cancer remains unsatisfactory for most patients because of local invasion and/or distant metastasis.[7] Thus, understanding more about the precise molecular biology and screening for useful biomarkers is critical for improving the therapy and overall prognosis of this disease.

Activating transcription factor 3 (ATF3), one of the ATF/-cyclic AMP response element-binding (CREB) family members, shares the common feature of this protein family, the basic-region leucine zipper domain, bZIP. It can bind to the ATF/CREB *cis*-regulatory element (5′-TGACGTCA-3′) and interact with other ATF/CREB proteins or other essential cellular proteins, such as p53 mediated by the bZIP domain.[8] Previous studies have demonstrated that ATF3 mainly behaves as an adaptive-response gene involved in the maintenance of genetic integrity and cellular homeostasis under stressed conditions.[9] Recently, increasing evidence indicates that ATF3 plays crucial and diverse roles in the development of cancer. It is aberrantly expressed in a wide range of human cancers, but its exact role in cancer remains unclear. For instance, it promotes tumorigenesis in breast and skin cancers and glioma, but has opposite and seemingly contradictory roles in colon and prostate cancers.[10–16] These discrepancies might reflect intrinsic differences in the cell models applied in different studies. The complex protein-protein interaction networks that ATF3 is involved in may also account for its context-dependent functions in human cancers. All these findings strongly suggest that ATF3 may be a novel therapeutic target for different cancers.

The expression, clinical significance, and biological functions of ATF3 in lung cancer remain largely unknown. In the present study, we determined the expression and clinical significance of ATF3 in lung cancer. ATF3 knockdown and overexpression assays were performed in H1299 and A549 cells. Alterations in *in vitro* malignant behaviors of lung cancer cells were comprehensively investigated.

Methods

Patients and tissues

Professor Fang Jing (Institute for Nutritional Sciences, SIBS, Chinese Academy of Sciences) generously donated fresh NSCLC tissues and paired non-cancerous normal lung tissues. All patients were diagnosed with histologically confirmed NSCLC and clinicopathological data were available. All of the patients provided written informed consent for the use of their samples. For total RNA and total protein extraction, tissues were immediately frozen by liquid nitrogen and stored at −80°C until used.

Cell culture and reagents

Human bronchial epithelial Beas-2B cells and lung cancer cells were cultured in 1640 or Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Sub-cell lines, high-metastatic L9981 and low-metastatic NL9980, were isolated and established from a human lung large cell carcinoma cell line.[17] The high-metastatic 95D and low-metastatic 95C were sublines of a human giant-cell lung carcinoma cell line.[18] All cell lines were obtained from the cell bank of the Tianjin Lung Cancer Institute (Tianjin, China).The antibody against ATF3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against β-actin was obtained from Sigma (St. Louis, MO, USA).

Short interference RNAs and plasmid transfections

For endogenous ATF3 knockdown, two independent short interference RNA (siRNA) oligos targeting ATF3 (siATF3-1 and siATF3-2) and control siRNA oligos (siNC) were obtained from GenePharma (Shanghai, China). The sequences of these oligos were: siATF3-1: CCUCUUUAU CCAACAGAUATT; siATF3-2: GGUUGUGCUUUCUAG CAAAUT; and siNC: UUCUCCGAACGUGUCACGUTT.

For exogenous ATF3 overexpression, the coding sequence of ATF3 was amplified from A549 cDNA by reverse transcription-PCR and inserted into the expression vector pcDNA3.1(+) using EcoRI and XhoI. The primer sequences were: forward: 5′-CGGAATTCATGATGCTTC AACACCCAGG-3′; reverse: 5′-CCCTCGAGTTAGCTCT GCAATGTTCCTTCTT-3′.

Transient transfection of cells was performed using LipofectAMINE-2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions.

Quantitative real-time PCR

RNA was extracted from the tissues or cells by TRIzol reagent (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on Applied Biosystems Step Two Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the comparative threshold cycle (Ct) quantization method. SYBR Premix Ex Taq (Takara, Tokyo, Japan) was used to detect and quantify the expression level of the target gene. β-actin was used as an internal control. ΔCt = Ct value of ATF3 − Ct value of β-actin. The primers were: ATF3 forward: 5′-CTCTGCGCTGGAATCAGTCA-3′; ATF3 reverse: 5′-TCGCCTCTTTTTCCTTTCATCT-3′; β-actin forward: 5′-GATCATTGCTCCTCCTGAGC-3′; and β-actin reverse: 5′-ACTCCTGCTTGCTGATCCAC-3′.

Immunoblotting

Immunoblotting was performed as previously described.[19] Briefly, tissues or cells were lysed on ice for 30 minutes in radioimmunoprecipitation assay buffer (Beyotime Biotechnology, Shanghai, China), supplemented with 1 mM phenylmethylsulfonyl fluoride. The supernatant was collected after centrifugation at 4°C, 12 800 rpm for 30 minutes. Equal amounts of protein were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitro-cellulose membrane. Proteins of interest were detected by immunoblotting using specific antibodies.

Immunohistochemistry staining

Immunohistochemistry staining of tissues was conducted as previously described.[20] Tissue samples were formaldehyde-fixed and processed by conventional paraffin-embedded method. The 5 μm thick sections were heat-immobilized, deparaffinized, and rehydrated. Endogenous peroxidases were blocked using 0.75% H_2O_2 in phosphate buffered saline (PBS) for 30 minutes. Antigen retrieval was performed by incubation in 10 mM citrate buffer (pH 6.0) for 10 minutes, followed by incubation in 5% BSA blocking buffer for an hour. The sections were incubated with primary anti-ATF3 antibody (1:200) at 4°C overnight. After washing the sections were then incubated with secondary antibody for an hour, and detected by incubation with streptavidin-horseradish peroxidase complex. The tissue sections were finally visualized by 3,3-diaminobenzidine and subsequently photographed under a microscope.

Cell proliferation assay

Cell proliferation was determined by counting cells under microscope using a hemocytometer. Cells were transfected with siRNAs or plasmids for 24 hours and then trypsinized and seeded into 12-well plates at a density of 4×10^5 cells. The cells were counted for the next three days. Counting experiments were independently repeated in triplicate.

Crystal violet staining assay

Cell viability was examined by means of crystal violet staining, as previously described.[19] Briefly, transfected cells in six-well plates were fixed in 4% paraformaldehyde for 20 minutes. After washing with PBS twice, the cells were stained with 0.1% crystal violet solution for 30 minutes. The plates were aspirated, washed, allowed to air dry, and photographed.

Cell cycle analysis

Cell cycle analysis was determined by propidium iodide (PI) staining, as previously described.[19] In brief, the cells were fixed with 70% ice-cold ethanol, followed by staining with freshly prepared nuclei staining buffer (0.1% Triton X-100 in PBS, 200 μg/mL of RNaseA, and 50 μg/mL of PI) for 20 minutes at 37°C. Cell-cycle histograms were generated using fluorescence-activated cell sorting analysis. The percentage of cells in the G0/G1, S, and G2/M phases were counted and compared.

Wound healing assay

Cell migration was determined by wound healing assay. Briefly, transfected cells were cultured in six-well plates to 100% confluence. A 1 mL plastic pipette tip was used to generate a clean wound area across the center of the well. Cell debris was removed by washing with PBS, and cells were allowed to migrate in the medium. The wound was assessed by microscope at 400× magnification at indicated time points, and the distance between the two edges of the wound was calculated from three different positions. At least four wound areas were investigated on each plate to quantify the migration.

Transwell assay

Cell invasion was determined by transwell assays. Twenty-four hours after transfection, the cells (3×10^4/well) in serum-free medium were seeded into the upper well of the transwell chamber coated with Matrigel (BD Bioscience, Mountain View, CA, USA) and allowed to invade toward the medium containing 10% FBS for 24 hours. The cells that reached the lower surface were fixed with methanol, followed by 0.1% crystal violet staining. The cells were counted by photographing the membrane through the microscope (400× magnification) from each chamber.

Statistical analysis

Statistical analyses were performed using a two-tailed Students t-test with GraphPad Prism 5 software (La Jolla, CA, USA). Protein quantification was performed using ImageJ software (NIH, Bethesda, MD, USA). Correlation between gene expression and clinicopathological features was performed by χ^2 test using SAS 8.02 software (SAS Institute Inc., Cary, NC, USA). Kaplan–Meier survival analysis was performed using a log rank test with original data from http://www.kmplot.com/. Differences of $P < 0.05$ were considered statistically significant. Data were represented as mean ± standard deviation (SD).

Results

Upregulation of activating transcription factor 3 (ATF3) in primary lung cancer tissues

To examine the ATF3 expression level in human lung cancer tissues, 64 pairs of NSCLC tissues and matched noncancerous normal lung tissues were collected. QRT-PCR detection revealed that ATF3 messenger RNA (mRNA) was highly expressed in NSCLC tissues and 53.13% of these exhibited more than twofold upregulation ($P < 0.001$, Fig 1a). Immunoblotting assay further demonstrated ATF3 protein upregulation in NSCLC tissues, as ATF3 protein was increased in most of the selected lung cancer tissues compared with the normal tissues (Fig 1b). Finally, we performed an immunohistochemical experiment, which indicated the same conclusion. ATF3 was indeed increased in NSCLC and manifested as brown particles in the tumor nuclei (Fig 1c).

High ATF3 levels correlated with advanced tumor grade, lymph node metastasis, and overall survival

To better understand the clinical significance of ATF3 expression in lung cancer, we correlated ATF3 mRNA expression in paired tissues with a series of clinicopathological features. As shown in Table 1, χ^2 analysis revealed high ATF3 expression in lung cancer was associated with advanced tumor grade ($P = 0.0266$) and lymph node metastasis ($P = 0.0110$), but not with patient gender, age, smoking history, or tumor type ($P > 0.05$). Using an online survival analysis tool (http://www.kmplot.com), we found that higher ATF3 expression predicted significantly poorer overall survival, as indicated by two independent probes targeting *ATF3* (Fig 2).[21]
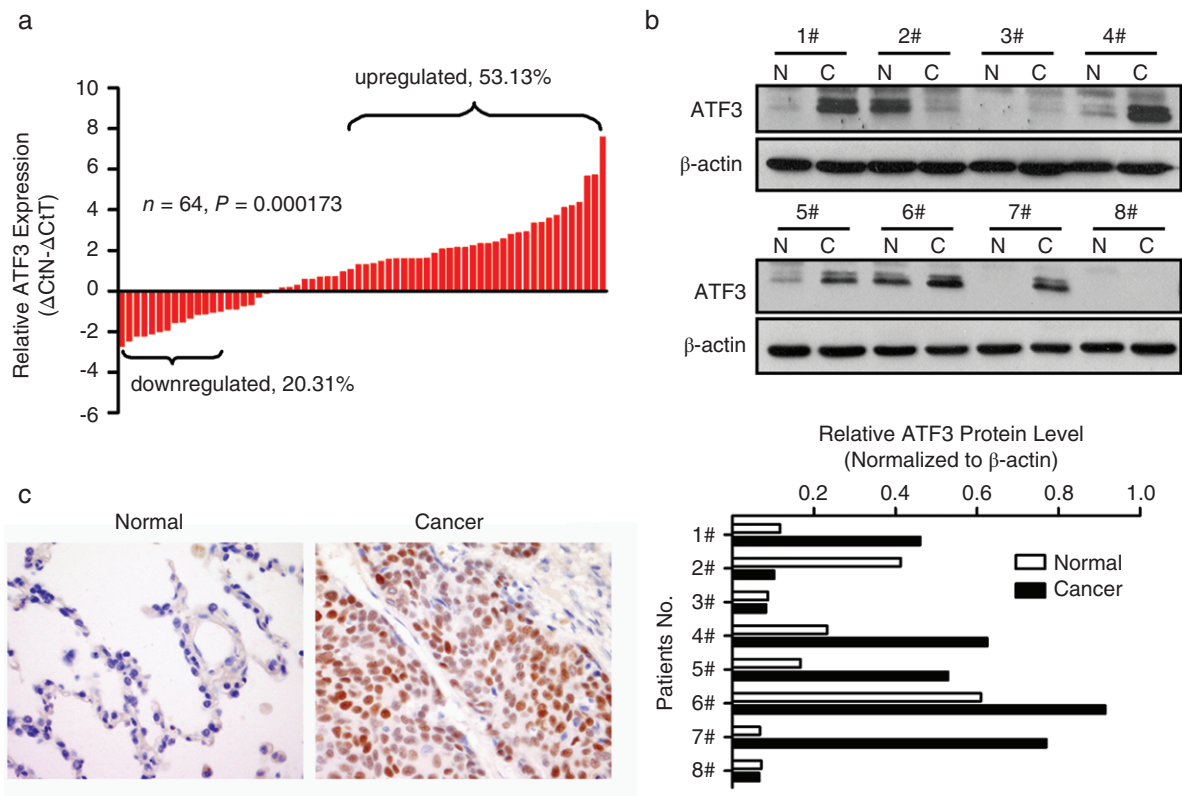
Figure 1 Activating transcription factor 3 (ATF3) expression was upregulated in non-small cell lung cancer (NSCLC) tissues. (**a**) Determination of ATF3 messenger RNA (mRNA) level by quantitative real-time PCR. ΔCtN: threshold cycle (Ct) value of β-actin was subtracted from the Ct value of ATF3 of paired normal tissue. ΔCtT: Ct value of β-actin was subtracted from that of ATF3 of NSCLC tissue. Bar value (ΔCtN − ΔCtT) represents the difference between ATF3 mRNA level in NSCLC and paired normal tissues. Bar value ≤−1 indicates that ATF3 expression was decreased in NSCLC tissues. Bar value ≥1 indicates that ATF3 expression of was increased in NSCLC tissues. (**b**) Determination of protein level of ATF3 by immunoblotting. The lower panel shows the quantitative results of ATF3 protein in the paired samples. (**c**) Immunohistochemical staining of paired samples with ATF3 antibody.

Table 1 Relationship between ATF3 expression levels in non-small cell lung cancer and patients' clinical and pathological features

| Clinical characteristics	Non-increased ($\Delta\Delta Ct \leq 1$) N = 30	Increased ($\Delta\Delta Ct > 1$) N = 34	Test of significance
Gender			
Male	19	22	$\chi^2 = 0.0130$
Female	11	12	$P = 0.9091$
Age			
< 60	18	17	$\chi^2 = 0.6432$
≥ 60	12	17	$P = 0.4226$
Smoking history			
Non-smoker	14	12	$\chi^2 = 0.8546$
Smoker	16	22	$P = 0.3553$
Tumor type			
Squamous cell lung carcinoma	14	14	
Lung adenocarcinoma	13	15	$\chi^2 = 4.2515$
Others	3	5	$P = 0.1193$
Tumor grade			
I + II	18	11	$\chi^2 = 4.9160$
III + IV	12	23	$P = 0.0266$
Lymph node metastasis			
No	13	5	$\chi^2 = 6.4612$
Yes	17	29	$P = 0.0110$

ATF3, activating transcription factor 3.

ATF3 upregulation in lung cancer cell lines

Activating transcription factor 3 expression in multiple lung cancer cell lines and one normal bronchial epithelial cell line was also examined. As shown in Figure 3a, ATF3 mRNA was overexpressed in most of the lung cancer cell lines. Among them, seven cell lines exhibited over 10-fold upregulation compared with normal bronchial epithelial Beas-2B cells. Moreover, ATF3 protein was also increased in six lung cancer cell lines (Fig 3b). This expression analysis revealed ATF3 upregulation in both lung cancer tissues and cell lines, which indicates its tumor promoting role in lung cancer cells.

ATF3 knockdown inhibited cell proliferation and cell cycle progression

Next, we performed RNAi experiments to investigate ATF3function. Two independent siRNAs targeting ATF3 and control siRNA (NC) were transfected in A549 and H1299 cells. ATF3 knockdown efficiency was first validated
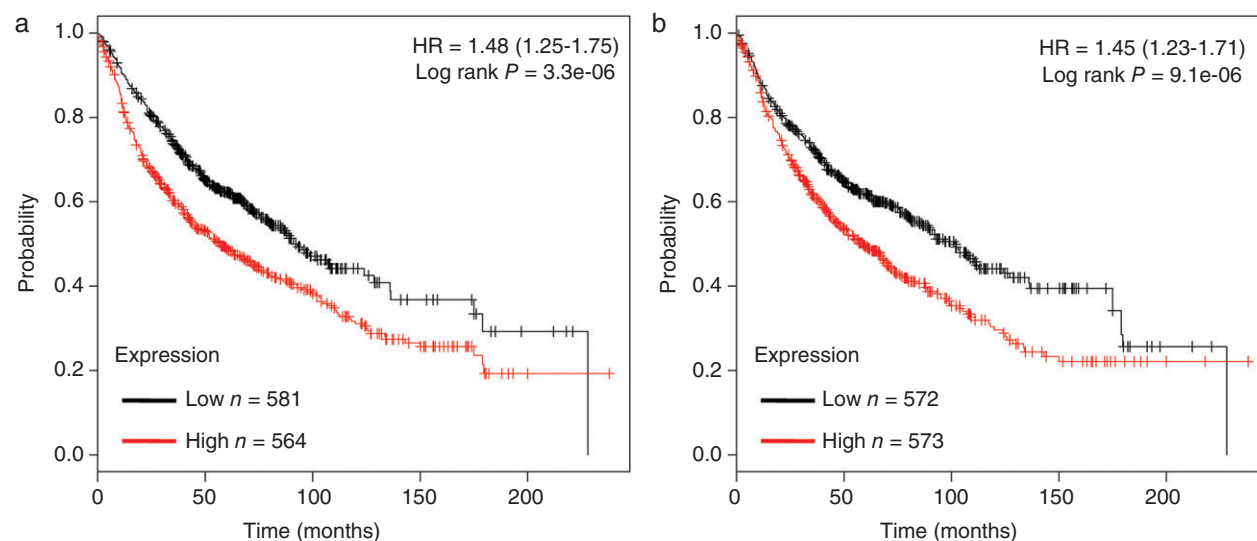
Figure 2 Higher activating transcription factor 3 (ATF3) expression predicted poor overall survival. Kaplan–Meier survival curves were derived for the *ATF3* gene in 1145 cases of lung cancer patients. Two independent probes were used to target *ATF3* gene (**a** and **b**).
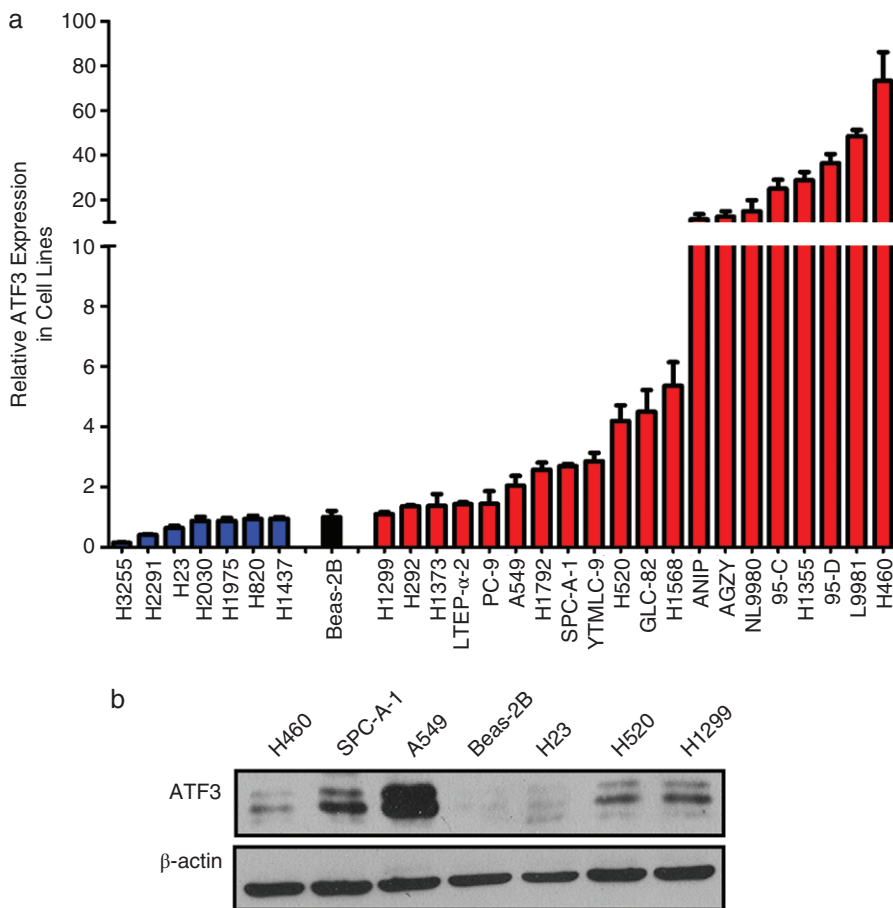
Figure 3 Activating transcription factor 3 (ATF3) expression was upregulated in lung cancer cells. (**a**) Determination of ATF3 messenger RNA by quantitative real-time PCR in human bronchial epithelial Beas-2B cells and 27 lung cancer cell lines. (**b**) Determination of ATF3 protein level by immunoblotting in human bronchial epithelial Beas-2B cells and six lung cancer cell lines.

to be effective (Fig 4a,b). To assess the effect of ATF3 inhibition on lung cancer cell proliferation, cell counting and crystal violet staining assays were performed. As shown in Figure 4c,d, we found that ATF3 knockdown led to markedly reduced proliferation and cell viability 48 hours after transfection. In addition, cell cycle analysis also revealed that ATF3 knockdown resulted in significant G1 phase arrest, which might explain the growth-inhibitory impact of ATF3-siRNAs in the two cell lines (Fig. 4e).

ATF3 knockdown impacted lung cancer cell migration and invasion

In order to determine the role of ATF3 in the metastasis potential of lung cancer cells, we analyzed ATF3 expression in two pairs of lung cancer cells with low or high metastatic ability (NL9980 vs. L9981; 95C vs. 95D). Results from qRT-PCR and immunoblotting demonstrated that ATF3 was overexpressed in the cells with relatively higher metastatic ability (L9981 and 95D), at both mRNA and protein levels (Fig 5a,b). Functionally, ATF3-siRNA transfection dramatically suppressed H1299 and A549 cell

migration and invasion, as gauged by the wound healing (Fig 5c) and transwell assays (Fig 5d).

ATF3 overexpression enhanced the proliferation, migration, and invasion abilities of lung cancer cells

RNAi mediated ATF3 knockdown proved the ATF3 oncogenic roles in lung cancer cells. We then overexpressed ATF3 in H1299 cells to determine the impact on cell proliferation, migration, and invasion exerted by forced expression of ATF3. The overexpression efficiency of ATF3 was validated to be effective (Fig 6a,b). Cell counting and crystal violet staining assays revealed that ATF3 overexpression dramatically reduced cell growth (Fig 6c,d). Moreover, wound healing and transwell invasion assays further verified that forced ATF3 expression significantly promoted lung cell migration and invasion (Fig 6e,f).

Discussion

Our data demonstrate that ATF3 expression is upregulated in human lung cancer tissues and cell lines. Higher ATF3
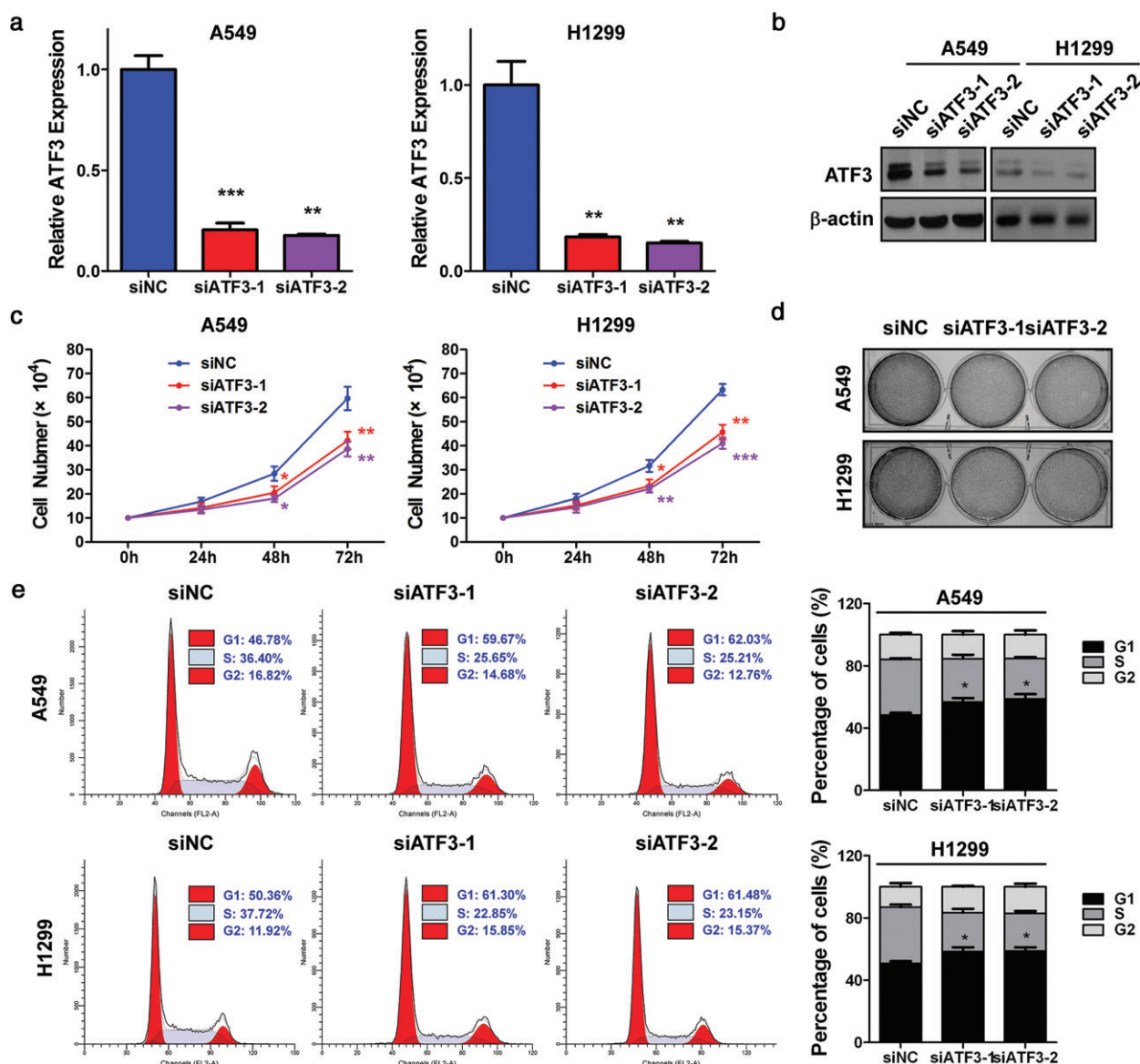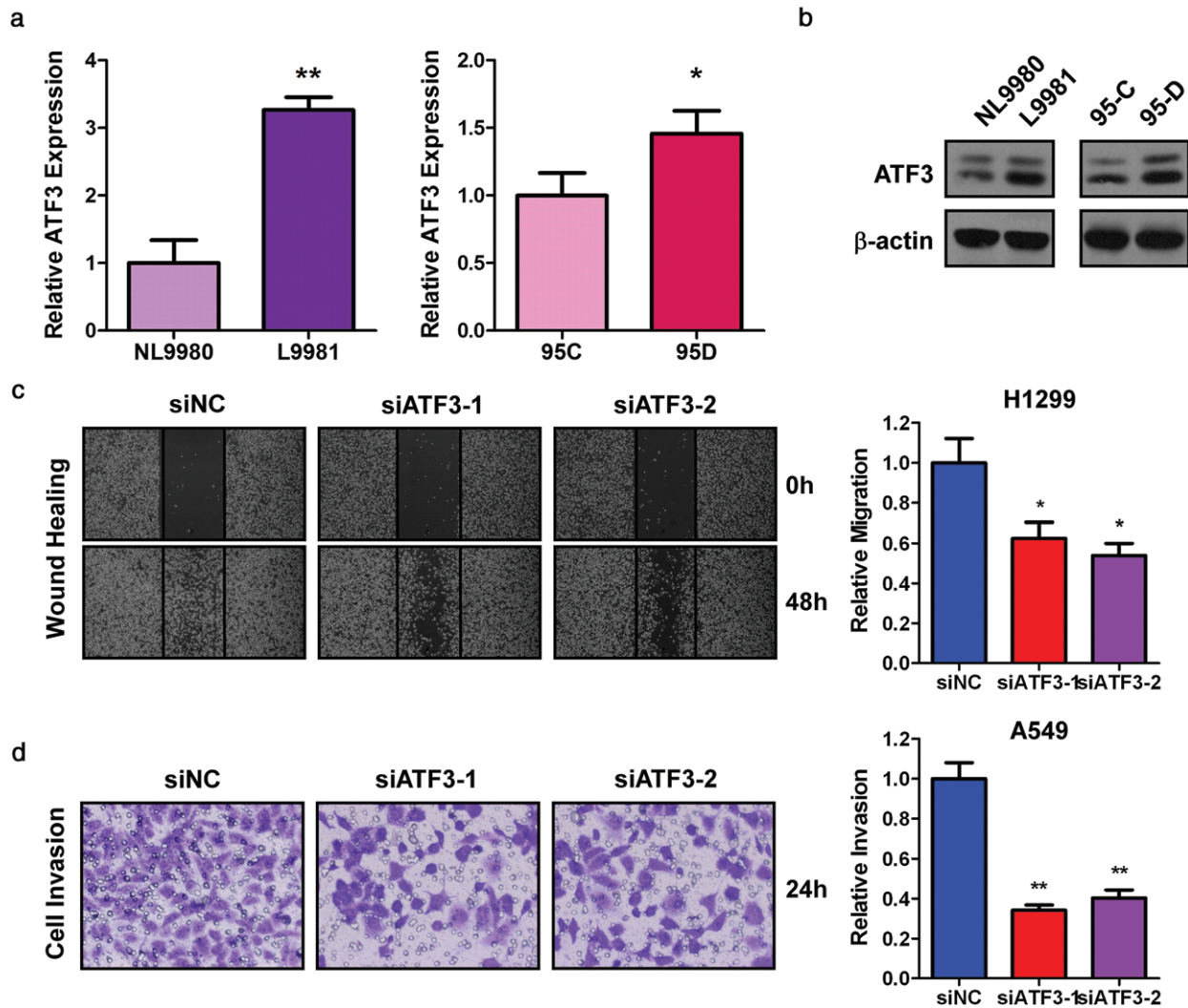
Figure 4 Knockdown of activating transcription factor 3 (ATF3) inhibited cell proliferation in the lung cancer cell. (**a**) A549 or H1299 cells were transfected with either control short interference RNA (siRNA) oligos or two ATF3 siRNA oligos. Forty-eight hours later, cells were harvested for quantitative real-time PCR. (**b**) ATF3 protein levels were detected by immunoblotting. (**c**) A549 or H1299 cells were transfected. After 24 hours, cells were trypsinized and seeded into 12-well plates. Cell proliferation assay was performed. (**d**) A549 or H1299 cells were transfected. After 72 hours, the cells were stained with crystal violet. (**e**) A549 or H1299 cells were transfected. After 48 hours, the cells were harvested for cell cycle assay. The left panel depicts DNA content while the right shows the quantitative results. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control.

expression was significantly correlated with advanced tumor grade, lymph node metastasis, and shorter overall survival. After performing a series of *in vitro* functional assays, we verified that ATF3 knockdown in two independent lung cancer cell lines (A549 and H1299) led to the inhibition of cell proliferation, migration, and invasion, whereas ATF3 overexpression did the opposite. Therefore, our results are supportive of earlier findings indicating that ATF3 might act

as an oncogene in carcinogenesis and provide the first experimental evidence associated with lung cancer.

The *ATF3* gene is located on human chromosome 1q32 within a region that is frequently amplified in solid tumors.[22] It is worth noting that ATF3 plays different roles in cancer progression, lying on the cell types, contexts, or microenvironment.[23,24] In normal tissues, ATF3 facilitates both cell growth and apoptosis,

Figure 5 Knockdown of activating transcription factor 3 (ATF3) impaired the migration and invasion of lung cancer cells. (**a**) Quantitative real-time PCR was performed to detect the ATF3 messenger RNA level in two pairs of lung cancer cells with low or high metastatic ability (NL9980 vs. L9981; 95C vs. 95D). (**b**) ATF3 protein levels were detected by immunoblotting. (**c**) Cells were transfected with either control short interference RNA (siRNA) oligos or two ATF3 siRNA oligos. Twenty-four hours later, cells were trypsinized and seeded into six-well plates. Migration abilities were assessed in H1299 cells by wound healing assay. The right panel shows the quantitative results. (**d**) Cells were transfected and invasion abilities in A549 cells were assessed by transwell assay. The right panel shows the quantitative results. *$P < 0.05$, **$P < 0.01$ versus control.

but also functions as either an oncogene or a tumor suppressor in neoplasms, which is highly relative to the specific tumor entity.[24–28] In regard to cell proliferation, previous research has shown that ATF3 could promote skin cancer cell proliferation through inhibiting p53 expression, and benefit laryngeal cancer cell growth, which might be correlated with cyclin D1 regulation.[10,29] ATF3 is regularly considered to be an inhibitor of cancer cell growth. Li *et al.* and Xie *et al.* revealed that ATF3 was reduced in esophageal squamous cell carcinoma, and ATF3 overexpression resulted in the inhibition of cell proliferation and invasion. This was associated with the inhibition of cyclin D1 and Twist,

which induces E-cadherin expression or the induction of p53 nuclear translocation and forms an ATF3/mouse double minute 2/matrix metalloproteinase-2 (MMP-2) complex that facilitates MMP-2 degradation.[30,31] Using *ATF3* knockout mice, ATF3 was found to be able to promote apoptosis and cell cycle arrest, and suppress Ras-mediated tumorigenesis partially via binding to the cyclin D1 promoter and repressing its transcription in mouse fibroblasts.[25] In a recent study using *Pten* knockout mice, loss of *ATF3* promoted the development of prostate cancer by activating protein kinase B signaling and increasing MMP-2/9 expression.[32] Invasion and metastasis are among the most important hallmarks of

Figure 6 Activating transcription factor 3 (ATF3) overexpression promoted the proliferation, migration, and invasion of lung cancer cells. (**a**) H1299 cells were transfected with either control plasmid or ATF3 expression vector. Twenty-four hours later, cells were harvested for quantitative real-time PCR. (**b**) ATF3 protein levels were detected by immunoblotting. (**c**) H1299 cells were transfected and after 24 hours, cells were trypsinized and seeded into 12-well plates. Cell proliferation assay was performed. (**d**) H1299 cells were transfected and after 48 hours, the cells were stained with crystal violet. (**e**) H1299 cells were transfected and 24 hours later, cells were trypsinized and seeded into six-well plates. Migration abilities were assessed by wound healing assay. The right panel shows the quantitative results. (**f**) H1299 cells were transfected and invasion abilities were assessed by transwell assay. The right panel shows the quantitative results. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
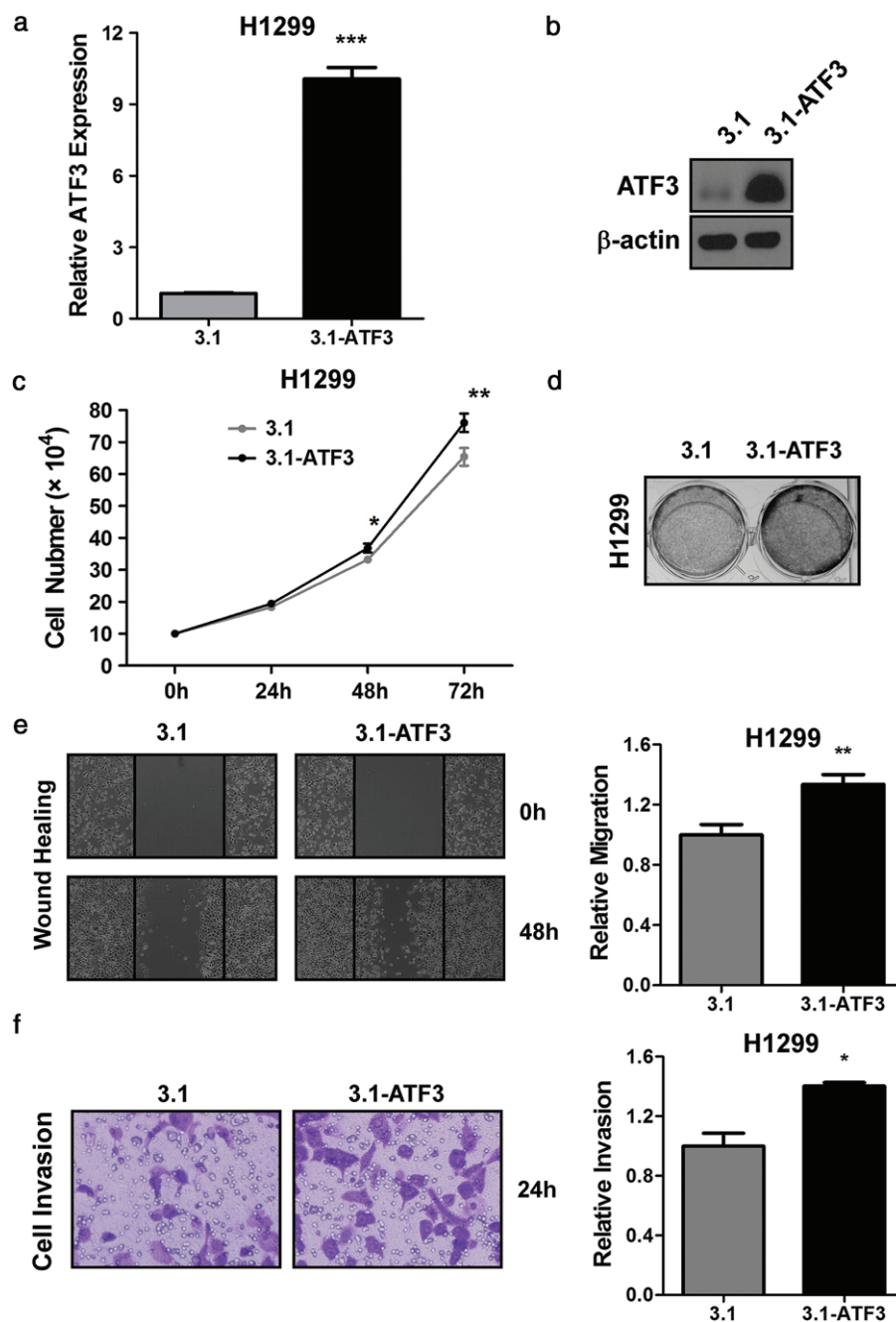
cancer.[33] It has been reported that ATF3 regulates cell invasion and metastasis in human ovarian and bladder cancers.[34,35] However, much still remains unknown about the complex roles ATF3 plays in the context of tumor progression.

Herein we focused on the biological roles of ATF3 in human lung cancer cells. A cohort of 64 pairs of lung cancer tissues was used to determine the mRNA expression of ATF3. We concluded that ATF3 plays an oncogenic role in lung cancer, which was inconsistent with an earlier report.[36]

As a downstream target of adenylate kinase-4, ATF3 was shown as a negative regulator for invasion activity in CL1-0 and CL1-5 cells.[36] We hypothesized that it was the cell context and sample size that made the direct opposite views. The correlation of ATF3 with clinicopathological features and overall survival in lung cancer was also examined. Our results indicated that ATF3 played a tumor promoting function in lung cancer. A limitation of this study is the lack of *in vivo* experiments. *In vitro* studies of ATF3 inhibition or overexpression provide sufficient clues for characterizing

the role of ATF3 in lung cancer cells; however, the underlying mechanisms still require elucidation.

Overall, our results show that ATF3 is significantly upregulated in lung cancer and is of important clinical significance. Critically, our loss and gain-of-function studies verified the tumor promoting activities of ATF3 in lung cancer cells, including promoting cell proliferation, migration, and invasion *in vitro*. Hence, ATF3 may be considered as a candidate therapeutic target for lung cancer and deserves more in-depth attention.

Acknowledgments

Disclosure

No authors report any conflict of interest.

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