FSTL1 suppresses tumor cell proliferation, invasion and survival in non-small cell lung cancer

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Abstract. Follistatin like-1 (FSTL1) is a secreted glycoprotein involved in a series of physiological and pathological processes. However, its contribution to the development of cancer, especially the pathogenesis of NSCLC, remains to be elucidated. We explored the expression, function, and molecular mechanism of FSTL1 in NSCLC. In this study, we detected the expression of FSTL1 in a panel of NSCLC cell lines and lung normal epithelial cell line by qRT-PCR and western blot analysis and found that FSTL1 was downregulated in NSCLC cells compared with normal control. Knockdown of FSTL1 with different shRNA sequences result in increased cell proliferation and cell migration, invasion and reduced cell apoptosis in A549 cell line with high FSTL1 endogenous level. FSTL1 overexpression in H446 cell line with low FSTL1 endogenous level suppressed cell proliferation and migration, invasion and increased cell apoptosis. Knockdown and overexpression of FSTL1 caused altered cell cycle. Reduced cell apoptosis was revealed in FSTL1 knockdown cells accompanied by increased FAS expression and decreased FASL, cleaved caspase-3 and -7 expression. By contrast, overexpression of FSTL1 caused reduced FAS level and increased activated caspase-3 and -7 expression, which may lead to increased cell apoptosis. Moreover, the changed migration and invasion ability in FSTL1 sufficient or deficient cells may be caused by alterations in MMP2, MMP3 and MMP9 expression. Altogether, our results revealed the critical tumor-suppression function of FSTL1 in NSCLC progression, suggesting that FSTL1 might be an important factor in NSCLC progression.

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Introduction

Lung cancer is one of the leading causes of cancer-related death in the world (1), and non-small cell lung cancer (NSCLC) accounts for ~80% of lung cancer caused deaths (2). The 5-year survival rate of NSCLC remains very low even though the mechanism studies of NSCLC progresses rapidly (3). In patients with NSCLC, regional lymph node, liver, contralateral lung, brain and bone marrow are the most commonly metastatic sites and resulted in the primary causes of death of NSCLC patients (4). Therefore, the major challenge in treating NSCLC is to find the underlying mechanisms regulating NSCLC metastasis.

Follistatin like-1 (FSTL1), also named TSC-36, located on chromosome 3 (5), is a BM-40/SPARC/osteonectin family protein which encodes a secreted glycoprotein (6). After the identification in mouse osteoblastic MC3T3E1 cells with TGF-β1 stimulation (7,8), FSTL1 was found to be downregulated by v-myc and v-ras overexpression cell lines, implying a possible role of FSTL1 in carcinogenesis (9). FSTL1 is expressed in almost all cell types and enriched in normal placenta, smooth muscle with various functions in different biological processes, especially in cell proliferation and migration (10-13). It has been reported that FSTL1 participated in the regulation of rheumatoid arthritis and other autoimmune diseases (14). FSTL1 can regulate cell cycle entry to improve cardiac function and cardio-renal communication (15). In the kidney, FSTL1 is expressed in the loop of Henle and protects the kidney from being attacked by acute nephrotoxic injury (16). Moreover, myocytes produce less FSTL1 to increase glomerular and tubulointerstitial damage in the kidney (17). In the process of lung morphogenesis, FSTL1 is essential for tracheal cartilage formation and alveolar maturation through BMP signaling (18).

The function of FSTL1 in cancer was investigated in many kinds of tumors such as lung cancer, colon cancer, stomach cancer, breast cancer (7), renal cell carcinoma (19), ovarian and endometrial carcinoma (20). Also, expression level of FSTL1 depends on the degree of malignancies in cancer patients. However the function of FSTL1 in cancer is controversial. Recent reports showed that high level of FSTL1 was associated

with poor prognosis of glioblastoma (21) and promoted cancer cell bone metastasis by inhibiting antitumor immune responses (22). In contrast, in colon, stomach, breast, kidney, and ovarian cancers, the expression of FSTL1 is decreased suggesting a role of tumor suppressor as well (7,20,23,24). In lung cancer cells, FSTL1 inhibition could promote mitotic cell death by inactivated Erk1/2 (25). In this study, we further explored the function of FSTL1 in NSCLC tumor cell proliferation, migration, invasion as well as apoptosis. Gain- and loss-of-function experiments demonstrated that FSTL1 suppressed tumor cell proliferation with altered the cell cycle. FSTL1 inhibited cell survival, migration and invasion of NSCLC cells. The key factors associated with cell apoptosis and invasion including FAS/FASL, caspases and MMPs were changed with FSTL1. Our results indicated the crucial functions of FSTL1 in NSCLC and suggested that FSTL1 might by a new important factor in NSCLC progression.

Materials and methods

Cell lines. The human NSCLC cell lines were purchased from ATCC. Beas-2b, H446, H460, A549 and H1299 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All the medium were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37˚C. All the cells were confirmed to be free from mycoplasma contamination.

Establishment of stable cell lines in which FSTL1 was overexpressed or knocked down. The targeted knockdown sequence was the following: SH1: 5'-tctgagaagttgaggcaaa-3'; SH4: 5'-gcagcaactacagtgaaatcc-3'. The negative sequence was 5'-gtagcgcggtgtattatac-3'. Lentiviral vectors encoding shRNA were constructed by Hanyin Co. (Shanghai, China). The recombinant lentiviruses (SH) and the negative control (NC) lentivirus (Hanyin Co.) were prepared and titered to 10^9 transfection units (TU)/ml. To obtain stable cell lines, cells were seeded in 6-well plates and infected with virus and polybrene the following day. Positive clones were selected with puromycin for 14 days to establish the stable cell lines. Additionally, the lentiviruses expressing the FSTL1 sequence (OE) and the negative control lentivirus (NC) were constructed by Hanyin Co. FSTL1-OE and control stable cell lines were then established. The efficiency of FSTL1 knockdown and overexpression was confirmed by qRT-PCR and western blot analysis.

Western blot analysis. Protein lysates (50 μg per lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After blocking with 5% fat-free milk, the membranes were incubated with primary antibodies (1:500 dilution) at 4˚C overnight, followed by horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution). Anti-human RBM8A (Santa Cruz, sc-32312), anti-human actin (Proteintech, HRP-60008), anti-human snail (Cell Signaling Technology, 3879), anti-human p-stat3 (Cell Signaling Technology, 9131), anti-human stat3 (Cell Signaling Technology, 12640), anti-human fibronectin (Sigma-Aldrich, F3648), anti-human vimentin (Cell Signaling Technology, 5741), anti-human Notch (Abways Technology, CY52444) and donkey anti-rabbit IgG (Cell Signaling Technology, 7074) were used in this study. Immune-reactive proteins were visualized by enhanced chemiluminescence (ECL).

Total RNA isolation and quantitative real-time PCR (qRT-PCR) analysis. Total RNA was isolated from NSCLC cancer cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen). cDNA was reverse transcripted from 1 mg total RNA. qRT-PCR was performed with the SYBR Premix Ex Taq (Takara, Dalian, China). PCR primers were as follows: F: 5'-TCGCATCATCCAGTGGCTGGAA-3', R: 5'-TCACTGGAGTCCAGGCGAGAAT-3'.

The cycling conditions were as initial denaturation at 95˚C for 5 min, and then 36 cycles of denaturation at 95˚C for 10 sec and annealing at 60˚C for 30 sec. The relative mRNA expression was calculated using the comparative Ct ($\Delta\Delta$Ct) method.

Cell Counting Kit-8 (CCK8) assay. Cells were seeded into 96-well culture plates (5x10^3 cells/well). At 24, 48, 72 and 96 h, 10 μl CCK-8 reagent (Beyotime Biotechnology) was added to each well and incubated for 4 h. Then the absorbance values were read at a wavelength of 450 nm using a microplate reader (SpectraMax 250; GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Scratch assay. The *in vitro* migration ability of NSCLC cells was assessed by scratch assay. Cells were seeded in 6-well plates and the monolayer was scratched with 10-μl pipette tips. The wound areas were photographed 0 and 20 h after scratching and measured using a caliper. The wound-closure percentages were calculated using the following formula: [1-(current wound size/initial wound size)] x 100.

Cell invasion assay. Cells were detached and re-suspended in a serum-free medium and seeded on the upper chamber of Matrigel-coated Transwell inserts with a pore size of 8 μm. The culture medium containing 10% FBS as a chemoattractant was added to the lower chamber. After 24-h incubation, the cells on the upper surface of the insert were gently removed with a cotton swab. Invading cells (lower surface of the insert) were fixed with 4% paraformaldehyde (Sigma-Aldrich), stained with crystal violet, and counted under a microscope. Five random microscopic fields were examined for each insert.

Flow cytometry analysis. Cells were seeded into 6-well plates at a density of 1x10^6 cells/well for 24 h. Subsequently, the cells were collected and stained with the ANXA5 (Annexin V)-PE apoptosis detection kit (4A Biotech Co. Ltd., FXP018-100) according to the manufacturer's instructions and analyzed by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA).

Statistical analyses. Unless stated otherwise, data are presented as mean ± SD in the figures. A Student's t-test was performed to compare the two groups of *in vitro* data. For more than two groups, we analyzed with one-way ANOVA followed by Tukey's multiple comparison test. All statistical tests were two-sided.

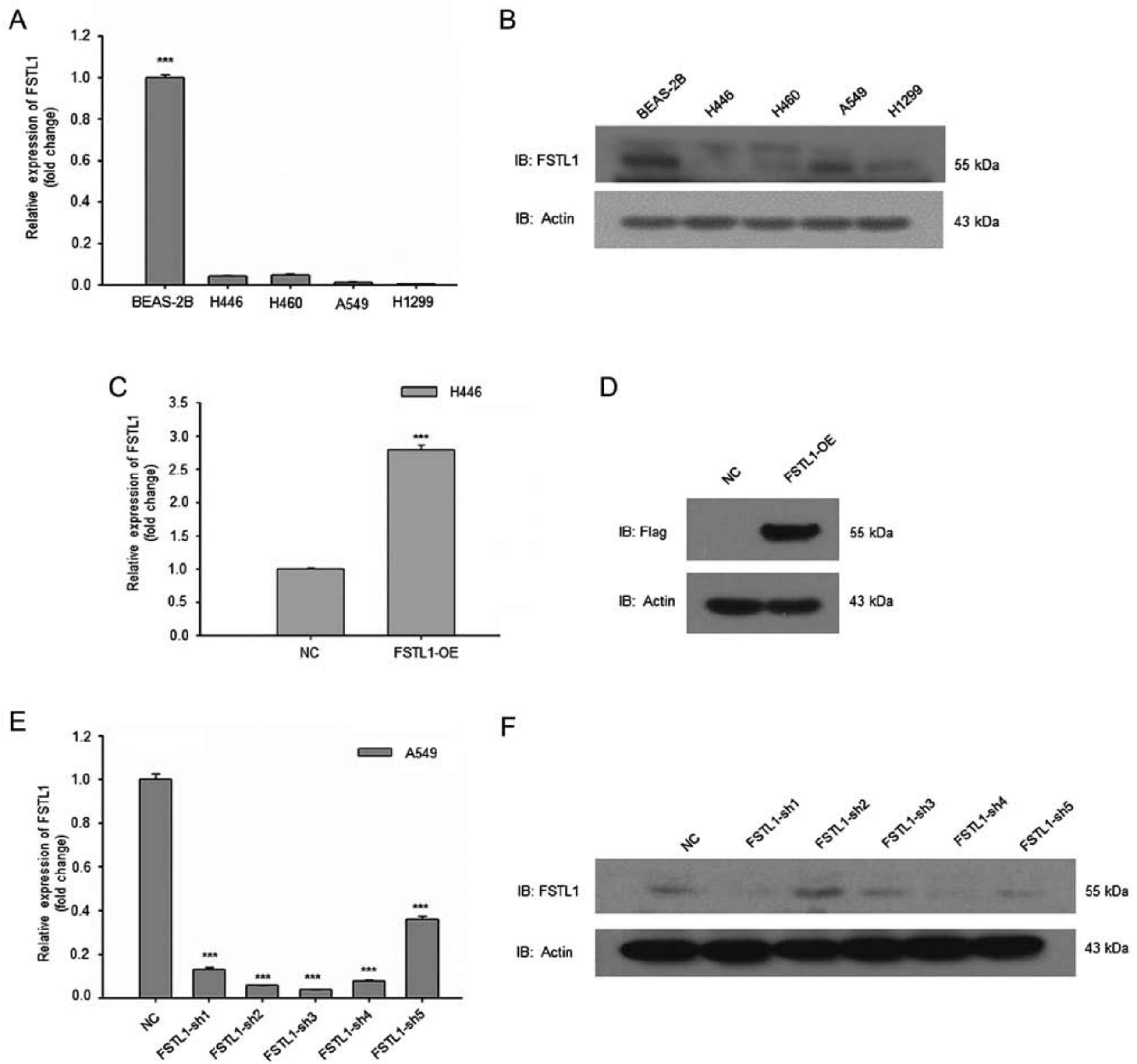

Figure 1. Expression of FSTL1 in lung cancer cells and lung normal epithelial cell line. qRT-PCR (A) and western blot analysis (B) of FSTL1 mRNA expression level in human lung normal epithelial cell line and NSCLC cell lines. Overexpression of FSTL1 in H446 cell line. FSTL1 expression was analysis with qRT-PCR (C) and western blot analysis (D). Knockdown of FSTL1 in A549 cell line with 5 different shRNA sequences. FSTL1 expression was analyzed with qRT-PCR (E) and western blot analysis (F). Student's t-test; N=3; error bars, ± SEM. ***P<0.001.

Results

FSTL1 is downregulated in NSCLC cells. In order to explore the function of FSTL1 in NSCLC, we collected an array of lung cancer cells and lung normal epithelial cell line, BEAS-2B. Expression of FSTL1 was examined by qRT-PCR and western blot analysis. As shown in Fig. 1A, the mRNA levels of FSTL1 in NSCLC cells were much lower than normal BEAS-2B cells. Consistently, the protein level of FSTL1 in BEAS-2B was higher than NSCLC cells (Fig. 1B). These results suggest that FSTL1 is downregulated in NSCLC cells.

We then constructed FSTL1 overexpression in H446 cells. Both RT-PCR and western blot analysis revealed the successful establishment of FSTL1 overexpression (Fig. 1C and D). Then FSTL1 expression was knocked down in A549 cells. The results of qRT-PCR and western blot analysis shown, FSTL1 was effectively suppressed by SH1 and SH4 (Fig. 1E and F).

FSTL1 reduced NSCLC cell proliferation with altered cell cycle. To analyze the function of FSTL1 in NSCLC cells, we examined the cell proliferation ability using CCK8. The results showed that A549 cells with FSTL1 knockdown proliferated faster than control cells (Fig. 2A). On the contrary, H446 cells with FSTL1 overexpression proliferated slower than control cells (Fig. 2B). In order to further clarify the function of FSTL1 in NSCLC cells, we examined the cell cycle of A549 cells with FSTL1 knockdown. As shown in Fig. 2C and D, the percentage of G1 phase in FSTL1 suppressed A549 cells was reduced while the percentage of G2 phase was elevated. However, the percentage of G1 phase in FSTL1 overexpressed

Figure 2. FSTL1 reduces NSCLC cell proliferation with altered cell cycle. Cell growth viability was assayed in A549 control cells (A549-NC) and FSTL1 knockdown A549 cells (A549-FSTL1-sh1, A549-FSTL1-sh4) (A), H446 control cells (H446-NC) and FSTL1 overexpression H446 cell line (H446-FSTL1-OE) (B) by CCK-8 assay at day 1, 2, 3, 4 and 5 time-points. (C) Cell cycle was assayed in A549 control and FSTL1 knockdown cells. (D) Quantification of the cell cycle in (C). (E) Cell cycle was assayed in H446 control and FSTL1 overexpressed cells. (F) Quantification of cell cycle in (E). Data are presented as mean ± SEM, ***P<0.001.

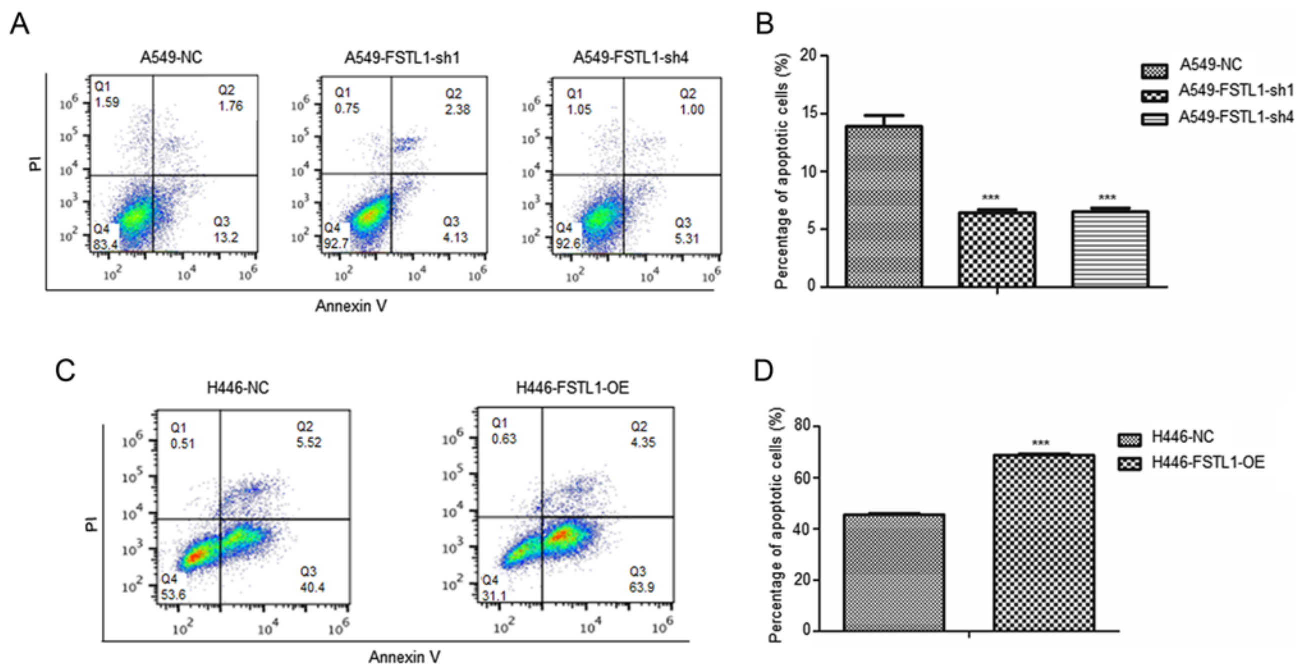

Figure 3. FSTL1 plays important function in NSCLC cell survival. (A) FACS analysis of apoptosis cells in A549 control and FSTL1 knockdown A549 cells. (B) Quantification of apoptotic cells in (A). Data are presented as mean ± SEM, ***P<0.001. (C) FACS analysis of apoptosis cells in H446 control and FSTL1 overexpressed H446 cells. (D) Quantification of apoptotic cells in (C). Data are presented as mean ± SEM, ***P<0.001.

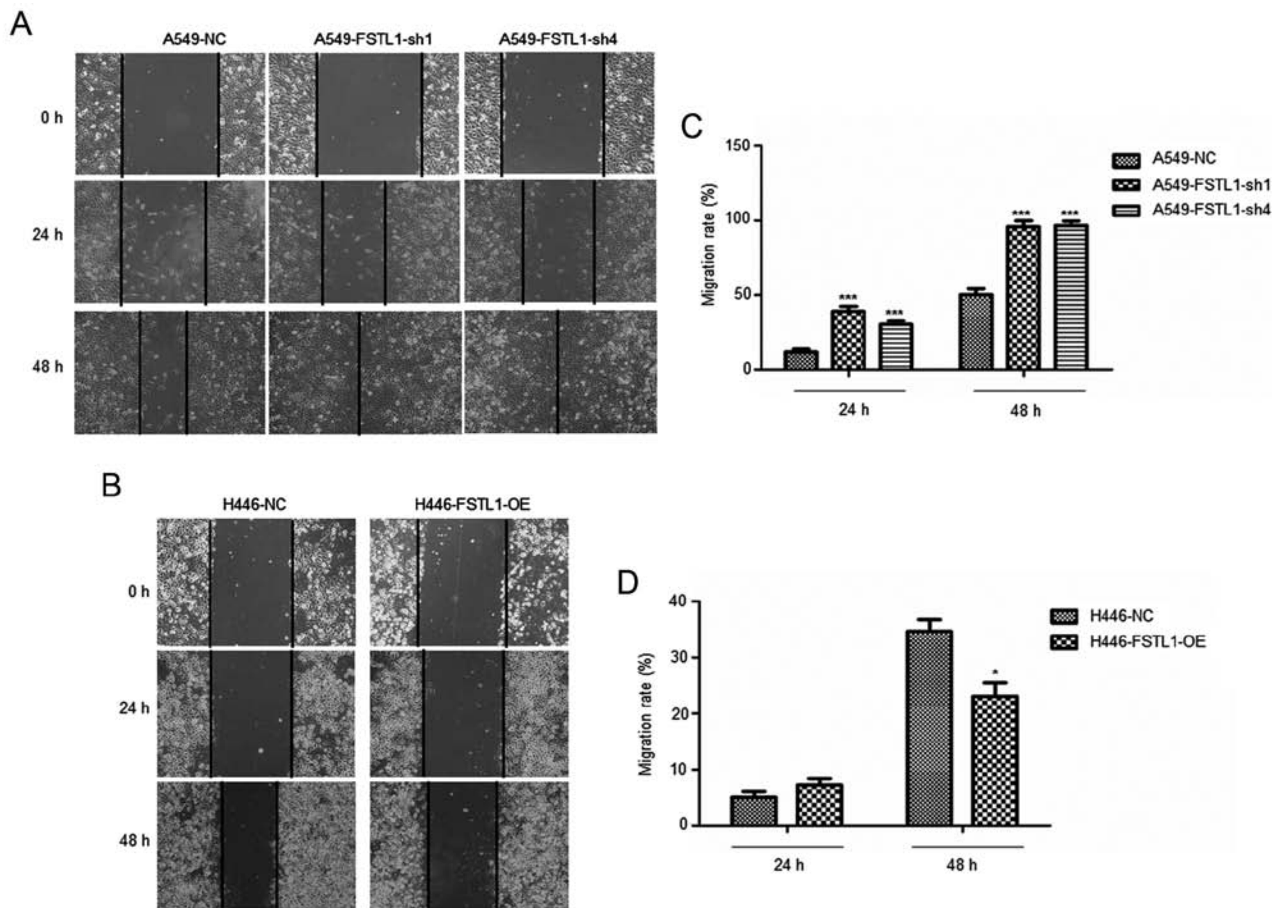

Figure 4. FSTL1 suppresses NSCLC cell migration. (A) Status of cell migration at 0-, 24- and 48-h time-points was detected by wound healing assay in A549 control and FSTL1 knockdown A549 cells. (B) Quantification of cell migration in (A). Data are presented as mean ± SEM, ***P<0.001. (C) Status of cell migration at 0-, 24- and 48-h time-points was detected by wound healing assay in H446 control and FSTL1 overexpression H446 cells. (D) Quantification of cell migration in (C). Data are presented as mean ± SEM, *P<0.05.

H446 cells was increased while the percentage of G2 phase was reduced (Fig. 2E and F).

We also examined cell apoptosis after changing FSTL1 expression. As shown in Fig. 3A and B, the percentage of apoptotic cells in FSTL1-suppressed A549 cells was reduced significantly. However, the percentage of apoptotic cells in FSTL1 overexpressed H446 cells was increased (Fig. 3C and D).

Taken together, these results indicate that FSTL1 reduced NSCLC cell proliferation with altered cell cycle and elevated apoptosis.

FSTL1 suppressed NSCLC cell migration and invasion. To investigate the effect of FSTL1 on NSCLC cell migration and invasion, control cells, FSTL1 knockdown cells were submitted to scratch assay. Our results showed that the migration ability of A549 cells was significantly increased upon FSTL1 knockdown (Fig. 4A and B). However, the FSTL1 overexpression strongly reduced the migration ability of H446 cells.

Further, the Transwell assay showed that the invasion ability of A549 cells was significantly increased after FSTL1 knockdown (Fig. 5A and B). On the contrary, ectopic expression of FSTL1 reduced the invasion of H446 cells (Fig. 5C and D). Thus, our results demonstrated that FSTL1 suppressed NSCLC cell migration and invasion.

FSTL1 regulates crucial factors in apoptosis and invasion. In order to elucidate the underlying mechanisms of FSTL1 mediated NSCLC cell apoptosis, we investigated the critical factors in cell apoptosis. We found a significant reduction of FASL, cleaved caspase-3 and -7, and upregulation of FAS and cleaved caspase-9 in FSTL1 knockdown A549 cells (Fig. 6A). On the contrary, in FSTL1 over expressed H446 cells, we observed an increase of FASL, cleaved caspase-3 and -7 (Fig. 6B) compared with control cells.

The important factors in cell migration and invasion were analyzed. MMP2, MMP3 and MMP9 were increased in FSTL1 knockdown A549 cells (Fig. 6C). However, MMP2, MMP3 and MMP9 were reduced in FSTL1 overexpressing H446 cells (Fig. 6D).

Taken together, our results demonstrated that FSTL1 might functioned as a tumor suppressor in NSCLC, including suppressing tumor cell proliferation, invasion and survival.

Discussion

FSTL1 has been found downregulated in many human cancer cell lines such as lung cancer, colon cancer, stomach cancer, breast cancer (7) and tumor patient samples like renal cell carcinoma (19), ovarian and endometrial carcinoma (20). Our studies revealed that FSTL1 is significantly downregulated in

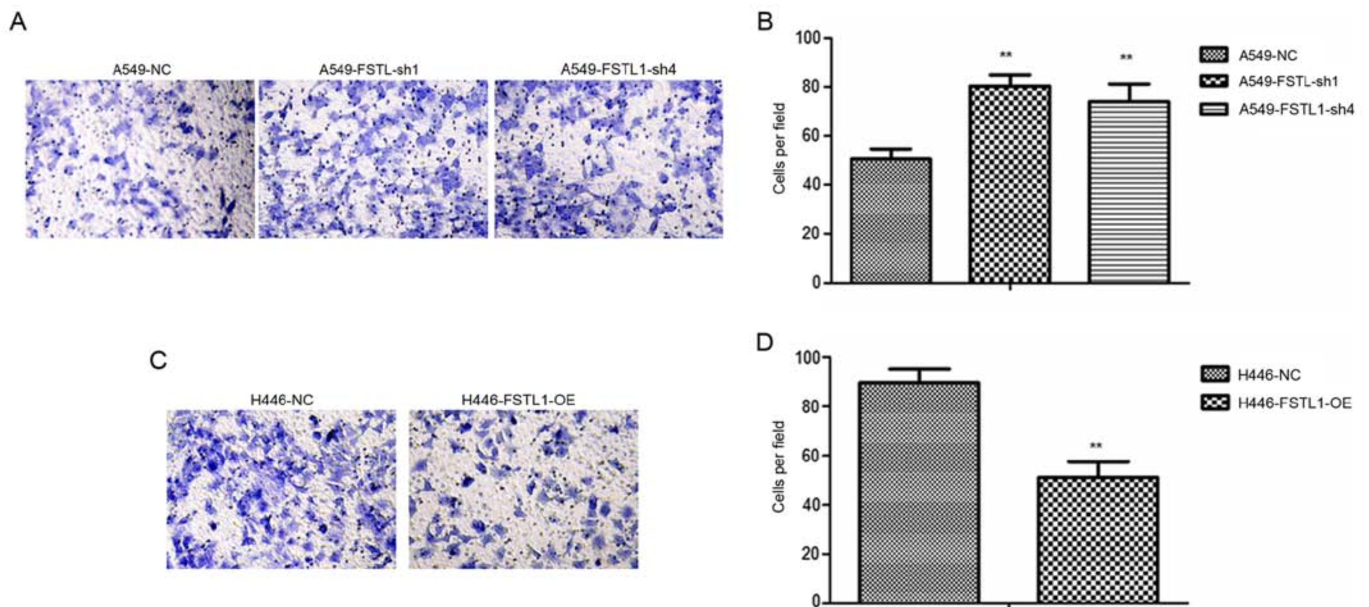

Figure 5. FSTL1 suppresses NSCLC cell invasion. (A) The invasion capacity of A549 control and FSTL1 knockdown A549 cells was detected by a Transwell invasive assay. (B) Quantification of cell invasion in (A). Data are presented as mean ± SEM, **P<0.01. (C) The invasion capacity of H446 control and FSTL1 overexpression H446 cells was detected by a Transwell invasion assay. (D) Quantification of cell invasion in (C). Data are presented as mean ± SEM, **P<0.01.

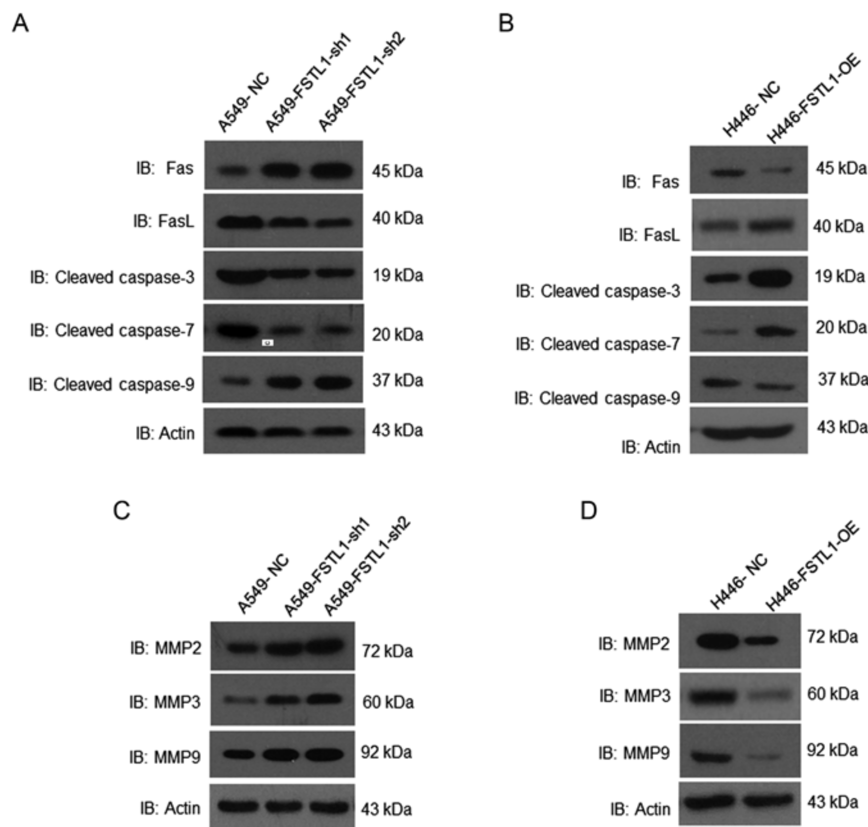

Figure 6. FSTL1 regulates crucial factors in apoptosis and invasion. Western blot analysis of apoptosis related protein level in A549 control, FSTL1 knockdown A549 cells (A) and H446 control, FSTL1 overexpression H446 cells (B). Western blot analysis of invasion related protein level in A549 control, FSTL1 knockdown A549 cells (C) and H446 control, FSTL1 overexpression H446 cells (D).

NSCLC cells. FSTL1 is also significantly downregulated in SCLC cell line H446. The relatively different levels of FSTL1 among these lung cancer cells might be associated with the heterogeneity of lung cancer. Being a tumor-suppressor gene, FSTL1 was first cloned from a mouse osteoblastic cell line and can be induced by TGF-β1 (26). In lung cancer cells, FSTL1 secretion can be influenced by connexin 43, which is also a tumor suppressor to suppress lung cancer cell invasion and

metastasis through regulating histone H3 and H4 acetylation and MMP-2 expression (27). Downregulation of MMP-2 was also detected upon FSTL1 expression (20). In nasopharyngeal cancer cell lines and tumor biopsies, FSTL1 gene was found downregulated by promoter hypermethylation (28). Overexpression of FSTL1 in nasopharyngeal cancer cell lines significantly suppressed tumor cell proliferation, migration and invasion ability and induced cell apoptosis.

The downregulated FSTL1 protein level in tumors was associated with downregulated interleukin 1β and tumor necrosis factor α, therefore, FSTL1 could activate macrophages and attenuate the immune evasion (28). Furthermore, FSTL1 deficiency induced the cellular arrest of G2/M phase in human lung cancer cells through the accumulation of cyclin-dependent kinase 1-cyclin B1 complex (25). Besides, based on the study of a multiple-organ metastasis model of human small cell lung cancer in natural killer cell-depleted SCID mice, FSTL1 was proved to play a critical role in the production of multiple-organ metastasis via inhibiting angiogenesis (29). FSTL1 has been found regulating many signaling pathways including AKT, NF-κB, SMAD and others (18,30-32). The receptor for FSTL1 has not been reported. However, FSTL1 can interact directly with BMP4 and influence the downstream signaling of BMP4/SMAD (18), suggesting that as a secreted glycoprotein, FSTL1 might trigger downstream signaling through binding to other ligands.

In this study, we found that FSTL1 was downregulated in NSCLC cells compared with normal control. FSTL1 overexpression suppressed tumor cell proliferation with altered cell cycle and induced cell apoptosis. In addition, FSTL1 inhibited cell survival, migration and invasion of NSCLC cells. The proteins associated with cell apoptosis and invasion including FAS/FASL, caspases and MMPs were regulated by FSTL1. Altogether, our results revealed the critical tumor-suppression function of FSTL1 in NSCLC progression, suggesting that FSTL1 might be an important factor in NSCLC progression.

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