Original Article
FRS2 promotes the progression of non-small cell lung cancer

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Abstract: Lung cancer is one of the leading causes for cancer mortality worldwide, and non-small cell lung cancer (NSCLC) accounts for the majority of all lung cancer cases. FRS2 is a member of the adaptor protein family that binds receptor tyrosine kinases, and is essential for the signaling cascades mediated by FGFR. In this study, we focused our research on the function of FRS2 in NSCLC, which has not been reported before. We found that FRS2 was upregulated in NSCLC tissues, and the expression levels of FRS2 in NSCLC were correlated with advanced stage, poor differentiation, and lymph node metastasis. Knockdown of FRS2 inhibited cell proliferation, migration and invasion in NSCLC cells lines. Importantly, knockdown of FRS2 inhibited ERK1/2 signaling in NSCLC cells, suggesting that FRS2 could affect NSCLC progression through the ERK1/2 signaling pathway.

Keywords: NSCLC, FRS2, ERK1/2, cell proliferation, cell migration

Introduction

Lung cancer is responsible for over 1.5 million deaths annually worldwide, and has become one of the leading causes of cancer mortality in both males and females [1]. Lung cancer can be histologically categorized into two major types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The concept of NSCLC encompasses a series of subtypes of cancers with different cell origin and morphology, including squamous cell carcinoma, adenocarcinoma, and large cell lung cancer. All subtypes of NSCLC combined account for 85% of all lung cancer cases. The clinical prognosis of NSCLC is poor despite recent scientific progress in cancer biology. This situation is primarily caused by the fact NSCLC cases are often diagnosed at advanced stages [2], leaving very limited options for the doctors to treat the disease. In addition, many patients with advanced NSCLC also suffer from relapse and ultimately develop chemotherapy resistance [3]. This situation prompts the urgent need for the research of pathophysiology of NSCLC and the development of effective therapeutic approaches.

Fibroblast growth factor receptor (FGFR) substrate 2 (FRS2) belongs to an adaptor protein family that contain phosphotyrosine-binding domain allowing for binding to receptor tyrosine kinases (RTK). FRS2 is essential for the signaling cascades mediated by FGFR [4]. The FGFR family RTKs were demonstrated to participate in the regulation of a variety of cellular processes including cell differentiation, proliferation, migration, and apoptosis [5, 6]. Aberrant regulation of FGFR signaling has been linked to cancer progression and was shown to affect cancer origination, angiogenesis, and metastasis of multiple cancer types, including breast cancer [7], renal cancer [8], myeloma [9], bladder cancer [10], myxoid liposarcoma [11] and gastric cancer [12]. Clinical studies have also indicated that dysfunction of FGFR signaling is correlated with poor cancer prognosis. Therefore, with these observations, FGFR signaling has become an attractive therapeutic target in human cancers.

FRS2 is located at the 12q15 locus in human genome. The amplification of this locus has been implicated in cancers. Cancer cell lines that harbor 12q15 amplification are sensitive to
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suppression of FRS2 [13]. In addition, 12q15 amplification was also found in high-grade liposarcoma cell lines through whole-exome sequencing, and these cells are also sensitive to FRS2 suppression induced by shRNAs [14, 15]. These studies supported an oncogenic role for FRS2.

In this study, we focused on the regulatory role of FRS2 in NSCLC, which has not been reported before. We found that FRS2 was upregulated in NSCLC tissues, and the expression levels of FRS2 in NSCLC were correlated with advanced stage, poor differentiation, and lymph node metastasis. Knockdown of FRS2 inhibited cell proliferation, migration and invasion in NSCLC cells. Importantly, knockdown of FRS2 inhibited ERK1/2 signaling in NSCLC cells, suggesting that FRS2 could affect NSCLC progression through the ERK1/2 signaling pathway.

Materials and methods

Human sample

A total of 48 non-small cell lung cancer (NSCLC) tissue samples and adjacent normal lung tissue samples (located >5 cm from the primary tumors) obtained by surgical resection at the Department of Thoracic Surgery, Hospital, were investigated in the present study. All patients hadn’t received radiation therapy or chemotherapy prior to the surgery. Tumor and normal lung tissues were harvested using sterile surgical instruments. Fresh tissue samples were immediately stored in liquid nitrogen for further using. This study protocol was approved by hospital ethical committee. All patients provided written informed consent for the use of their medical records and tissue specimens for research purposes. Clinicopathological information of the patients was obtained from patient records, which were summarized in Table 1.

Immunohistochemistry

Paraffin-embedded tissues were cut into 5 μm sections for dewaxing and rehydration. Immunohistochemistry was performed after microwave heating-based antigen retrieval in 0.1 M citrate buffer followed by incubation with 3% H2O2 at room temperature for 30 min, 10% goat serum at room temperature for 30 min, primary antibody against FRS2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, biotin-labeled secondary antibody (Santa Cruz Biotechnology) at room temperature for 30 min, and freshly prepared 3,3’-diaminobenzidine (Boster, Wuhan, China) for color development for 5 min. Sections were then thoroughly rinsed with water, restained with hematoxylin, dehydrated, cleared in xylene and mounted with a coverslip.

RT-PCR

Total RNA was extracted using miRNAeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions First-strand cDNA was synthesized from 1 μg of total RNA according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). qPCR was performed by SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) for the expression of FRS2. Expression of FRS2 gene was measured using the 2-ΔΔCt method. The primers are: FRS2 forward, 5'-CTGCCGAGGAGCACTGTCC-3'; reverse, 5'-CACGTTTGGCGTATTGAAAGTC-3'; β-actin forward, 5'-TTCTACAAATGAGCTGC-
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**Figure 1.** FRS2 was upregulated in NSCLC. A. Immunohistochemistry staining of FRS2 in normal adjacent lung tissues and NSCLC tissues. B. Relative mRNA levels of FRS2 in 48 NSCLC tissues, compared with adjacent normal lung tissues. ***P<0.001.

**Figure 2.** Establishment of FRS2 knockdown NSCLC cells. NSCLC A549 and H1975 cells were transfected with FRS2 siRNA or control siRNA for 48 hours. Cell lysates were subjected to Western blot for FRS2. The intensity of FRS2 bands was quantified by densitometry. ***P<0.001 compared with control siRNA transfected NSCLC cells.

GTGTG-3'; reverse, 5'-GGGG-TGTGAAGGTCTCAA-3'.

**Cell lines and cell culture**

Human NSCLC cell lines (A549 and H1975) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in RPMI-1640 medium (Gibco Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen), 100 U/mL penicillin and 100 U/mL streptomycin. The cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

**Cell transfection**

NSCLC cells were seeded in a 6-well plate at 5x10⁵ cells per well overnight for attaching. Cells were followed by transfection with Lipofectamine® RNAiMAX Reagent (Invitrogen) with a final according to the manufacturer’s instructions. The final concentrations of siRNA for the transfection were 10 µM. Transfection efficiencies were determined by western blot 48 h post-transfection.

**Western blot**

NSCLC cells were transfected with FRS2 or control siRNA. 48 hours later, cells washed
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Figure 3. Knockdown of FRS2 inhibited NSCLC cell proliferation. FRS2 Knockdown A549 and H1975 cells were subjected to MTT cell proliferation assays. Absorbance at 570 nm was measured on day 1, 2, 3 and 4. *P<0.05, **P<0.01 compared with control siRNA transfected NSCLC cells.

Figure 4. Knockdown of FRS2 inhibited NSCLC cell migration. FRS2 Knockdown A549 and H1975 cells were subjected to wound healing assays. Relative percentage of wound healing was quantified. *P<0.05 compared with control siRNA transfected NSCLC cells.

with PBS and lysed with RIPA lysis buffer to extract the total protein. The protein concentration of cell lysates was determined by BCA Protein Assay Kit (Beyotime, Beijing, China). 30 µg sample extract was resolved on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membrane (PVDF, Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h with 5% BSA in Tris-buffered saline containing 0.05% Tween (TBST). Membranes were incubated overnight at 4°C with primary antibody (anti-FRS2, anti-β-actin, anti-p-ERK and anti-ERK) (Santa Cruz Biotechnology), and then washed and incubated with secondary antibody, horse radish peroxidase (HRP) labeled goat anti-rabbit or anti-mouse immunoglobulin G (IgG) (Santa Cruz Biotechnology). All antibodies were dissolved in Tris-buffered saline with Tween (TBST) in a ratio of 1:1000, and finally protein expression was visualized by Enhanced Chemiluminescence (ECL, Beyotime).

**MTT assay**

NSCLC cells with a number of 2.5×10^3/well were seeded into 96-well plates and incubated...
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overnight. Transfection was then performed. At 48 h after transfection, each well was treated with 20 μL MTT solution and incubated sequentially at 37°C. After the incubation for 4 h, 100 μL of DMSO was added to dissolve the crystals. Then the absorbance of each well in culture plate was measured at 570 nm.

Cell migration assay

Wound healing assay was used to study the ability of NSCLC cell migration. Cells were seeded in six-well tissue culture dishes overnight in complete medium and grew to 90% confluence. The cell layer was scratched with 200 μl sterile pipette tip. Afterwards, the plates were washed twice and incubated for 24 h. At the bottom side of each dish, two arbitrary places were marked where the width of the wound was measured with an inverted microscope (Olympus, Japan). Wound healing was followed by acquiring digital image.

Cell invasion assay

The NSCLC cellular invasive ability was tested using a Matrigel Invasion Chamber (8-μm pore size, Corning, Corning, NY, USA) according to the manufacturer's protocol. Briefly, cells (1×10⁶ cells in 0.2 mL media) were incubated into the upper chambers of the system, the bottom wells in the system were filled with RPMI 1640 supplemented with 10% fetal bovine serum as a chemo-attractant and then incubated at 37°C for 24 h. Non-penetrating cells on the upper surface of the filter were removed using a cotton swab. The cells that had migrated through the Matrigel were fixed with methanol, stained using 0.1% crystal violet and counted under a microscope (Olympus).

Statistical analysis

The data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. The values for the capillary tube formation and luciferase activity assays were obtained from three independent experiments performed in duplicate. The differences between groups were analyzed using Student’s t test when only two groups were compared or using a one-way ANOVA when more than two groups were compared. All statistical tests were two-sided. The differences were considered statistically significant at P<0.05. All analyses were performed using SPSS software for windows (version 19.0, SPSS Inc., Chicago, IL, USA).

Results

FRS2 was upregulated in NSCLC

In order to investigate the role of FRS2 in NSCLC development, we first compared the expression levels of FRS2 in NSCLC tissues versus adjacent normal lung tissues. We found that the
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protein levels of FRS2 were significantly upregulated in NSCLC tissues, as demonstrated in the immunohistochemistry staining (Figure 1A). Consistently, by qPCR assay, we determined that the mRNA levels of FRS2 were also elevated in NSCLC tissues (Figure 1B). In addition, clinical statistics also demonstrated that high FRS2 levels were significantly correlated with advanced stage, poor differentiation, and lymph node metastasis of NSCLC (Table 1).

Knockdown of FRS2 inhibited NSCLC cell proliferation

We next used two independent NSCLC cell lines (A549 and H1975) to examine the function of FRS2 on cellular processes. By siRNA transfection, we successfully knocked down the expression of FRS2 in these two cell lines (Figure 2). We found that in both cell lines, knockdown of FRS2 led to significantly decreased cell proliferation, as evidenced by MTT assay (Figure 3).

Knockdown of FRS2 inhibited ERK1/2 signaling in NSCLC

FRS2 has been reported to regulate ERK1/2 signaling in other cancer types. Therefore we sought to determine whether FRS2 regulated ERK1/2 signaling in NSCLC. We performed Western blot analysis to detect the total and the phosphorylated levels of ERK1/2 in NSCLC cells, and we found that FRS2 knockdown led to inhibition of ERK1/2 phosphorylation in both A549 and H1975 cells, whereas the total protein levels of ERK1/2 did not change in both cell lines (Figure 6). These results indicated that FRS2 inhibited ERK1/2 signaling in NSCLC, and suggested that FRS2 could affect NSCLC cell proliferation and migration through the ERK1/2 signaling pathway.
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Discussion

In this study, we investigated the functional association between FRS2 and the progression of NSCLC. We demonstrated that the expression levels of FRS2 were elevated in NSCLC and were correlated with pathological stage and metastasis of cancers. We also found that knockdown of FRS2 inhibited the proliferation, migration and invasion of NSCLC cells. Importantly, we found that the ERK1/2 signaling pathways, which play essential roles in cancer development, were positively regulated by FRS2 in NSCLC cells.

FRS2 is essential for FGFR mediated signaling pathways. Dysfunction of FGFR signaling was associated with cancer progression and was shown to affect cancer origination, angiogenesis, and metastasis of multiple cancer types. Clinical studies have also indicated that dysfunction of FGFR signaling is correlated with poor cancer prognosis [7-12]. FRS2 itself was also shown to regulate the malignant progression of a variety of cancer types, but no research has been carried out regarding the roles of FRS2 in NSCLC [13-15]. Our study for the first time confirmed the relationship between FRS2 expression and the progression of NSCLC.

In our study, we found that the expression levels of FRS2 were positively correlated with the activity of the ERK1/2 signaling pathway. Indeed, previous study has provided a model by which FRS2 regulate the activity of ERK1/2 signaling. The activation of receptor tyrosine kinases including FGFR induces the phosphorylation of the FRS2 at tyrosine residues, thereby allowing binding to Grb2, an SH2 domain-containing adaptor protein, and Shp2, a tyrosine phosphatase. Shp2 subsequently activates the ERK1/2 pathway, and Grb2 can also activate the ERK1/2 pathway through binding to SOS via its SH3 domain [16, 17].

The ERK1/2 signaling pathway play essential roles in many aspects of cancer development, including regulating cell survival and apoptosis, cell proliferation, cell cycle progression, and cell matrix interaction [18-20]. It has also been reported by previous studies that activation of the ERK1/2 signaling pathways was associated with drug resistance in lung cancer [21, 22]. Therefore, it is reasonable to hypothesize that the mechanism of FRS2 regulating NSCLC progression could be via modulating ERK1/2 signaling. However, our study could not exclude the possibility that FRS2 regulated NSCLC development through other mediators besides ERK1/2.

Disclosure of conflict of interest

None.

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