MFAP2 contributes to the proliferation and motility of lung cancer cells via Wnt/β-catenin pathway

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Abstract

Purpose: To measure microfibrillar-associated protein 2 (MFAP2) expression levels in lung cancer and identify its role in lung cancer.

Methods: Immunoblots and PCR were used to determine the expression of MFAP2 in lung cancer cell lines. The effects of MFAP2 on the viability and apoptosis of lung cancer cells were evaluated by CCK-8, colony formation, and flow cytometry (FCM) assays, while wound healing and Transwell assays were used to assess the effects of MFAP2 on the motility and epithelial-mesenchymal transition (EMT) of lung cancer cells. Immunoblot assays were also performed to determine the effect of MFAP2 on the Wnt/β-catenin axis in lung cancer cells.

Results: MFAP2 was highly expressed in lung cancer cells. Depletion of MFAP2 suppressed the viability and stimulated apoptosis in these cells (p < 0.01). In addition, knockdown of MFAP2 suppressed the motility and EMT of lung cancer cells (p < 0.01). MFAP2 mediated the Wnt/β-catenin axis and affected the viability and motility of lung cancer cells.

Conclusion: MFAP2 is a promising target for lung cancer treatment.

Keywords: Microfibrillar-associated protein 2 (MFAP2), Lung cancer, Viability, Motility, Wnt/β-catenin pathway

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in males [1]. In China, the mortality of lung cancer is rising rapidly [2]. Lung cancer is also a heterogeneous disease [2]. Lung cancer is correlated with tobacco exposure and also with air pollution, genetic factors, viruses, diet, and obesity [3]. Although the 5-year survival of early stage lung cancer is high [4], in-depth studies and the development of several new targeted drugs for lung cancer remains at a low level [5]. It is therefore important to identify new biological targets for early diagnosis and treatment, to increase the survival of patients [6].

Microfibrillar-associated proteins (MFAPs) are a series of glycoproteins in the extracellular matrix, comprising a total of five subfamily members [7]. Microfibrillar-associated protein 2 (MFAP2), a member of the MFAP family, has attracted much attention in recent years because it can up-
MFAP2 affects several types of tumors [9]. For example, MFAP2 is highly expressed in papillary thyroid carcinoma, promotes the progression of cancer cells, and can be used as a potential biomarker and molecular target [9]. MFAP2 promotes the epithelial-mesenchymal transition (EMT) of gastric cancer cells by upregulating the transforming growth factor beta/SMad2/3 signaling pathway [10]. MFAP2 promotes the proliferation and invasion of hepatocellular carcinoma (HCC) cells and can be used as a prognostic marker of HCC and a target of immunotherapy [11]. MFAP2 can also promote the processes of melanoma cells via the Wnt/β-catenin axis [12]. It has been reported that MFAP2 was associated with lung function, and the expression of MFAP2 in lung cancer is higher than normal tissues as shown in The Cancer Genome Atlas (TCGA) database [13]. However, the potential role of MFAP2 in lung cancer progression remains unclear.

In this study, the expression of MFAP2 in human lung cancer cells and its role in the progression of lung cancer were investigated.

**METHODS**

**Antibodies, primers, and plasmids**

Anti-MFAP2 (1:500 dilution, ab231344; Abcam, Cambridge, UK), anti-E-cadherin (1:1,000 dilution, ab76055; Abcam), anti-N-cadherin (1:1,000 dilution, ab76011; Abcam), anti-Snail (1:500 dilution, ab216347; Abcam), anti-matrix metalloproteinase 2 (MMP2) (1:1,000 dilution, ab92536; Abcam), anti-MMP9 (1:1,000 dilution, ab76003; Abcam), anti-β-catenin (1:1,000 dilution, ab32572; Abcam), anti-glycogen synthase kinase 3β (GSK-3β) (1:1,000 dilution, ab32391; Abcam), anti-p-GSK-3β (1:500 dilution, ab75814; Abcam), and anti-β-actin (1:2,000 dilution, 60008-1-Ig; Proteintech, Rosemont, IL, USA) were used.

The quantitative PCR primer sequences of MFAP2 were the following: forward, 5’-TCCGCGGTGTACGTACATT-3’ and reverse, 5’-TCCGCGGTGTACGTACATT-3’. The quantitative PCR primer sequences of GAPDH were the following: 5’-TCCGCGGTGTACGTACATT-3’ and 5’-TCCGCGGTGTACGTACATT-3’.

The siRNA of MFAP2 and control siRNA were obtained from Ribio (Guangzhuo, China).

**Cell culture**

Normal lung epithelial cell lines BEAS-2B, A549, PC-9, NCI-H520, and NCI-H1703 were obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in DMEM supplemented with 10 % fetal bovine serum and incubated at 37 °C in a 5 % CO2 incubator.

**Quantitative PCR**

To extract total RNA from cells, TRizol® reagent (Invitrogen, Carlsbad, CA, USA) was used. Total RNA was reverse transcribed into cDNA at 42 ºC for 1 h using M-MLV reverse transcriptase (Promega, Madison, WI, USA), including 5 µL M-MLV 5× reaction buffer, 1.25 µL of 10 nM dNTP, 25 units of Recombinant RNasin® ribonuclease inhibitor, 200 units of M-MLV RT, and nuclease-free water to a final volume 25 µL. The qPCR was then performed using the SYBR Ex Taq kit (Takara Bio, Shiga, Japan) according to the manufacturer’s protocol. MFAP2 expression levels were normalized to the relative level of GAPDH. The following thermocycling conditions were used: initial denaturation at 95 ºC for 3 min; followed by 30 cycles of denaturation at 95 ºC for 30 s, annealing at 58ºC for 30 s, and extension at 72 ºC for 30 s.

**Immunoblot assay**

Cells were lysed with lysis buffer (RIPA; Beyotime, Beijing, China). Proteins were separated using 10 % SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA, USA). The membranes were then blocked using 5 % dry milk in TBST buffer and incubated with the indicated antibodies in TBST buffer for 2 h at room temperature. After washing with TBST three times, the membranes were treated with the secondary antibodies in TBST buffer for 2 h at room temperature for 45 min. Each blot was then visualized using an ECL kit (GE, Midrand, SA).

**Cell viability assay**

For CCK-8 assays, lung cancer cells were plated in 96-well plates (1,000 cells per well) and maintained in complete growth media for 24 h at 37°C. Cells were then exposed to CCK-8 reagent at 37 °C for 1.5 h. The relative cell viability was assessed using a microplate spectrophotometer at 450 nm (Bio-Rad, Hercules, CA, USA).

For the colony formation assay, lung cancer cells were plated into 24-well plates (1,000 cells per well) and maintained in complete growth media.
for 14 d at 37 °C. Subsequently, the cells were incubated with 0.2 % Crystal Violet and washed. Then the cells were photographed using a fluorescence microscope (Zeiss, Oberkochen, Germany).

**Cell apoptosis assay**

After transfection, the cells were washed for 48 h with phosphate-buffered saline. Subsequently, the cells were fixed with precooled 70 % ethanol at -20 °C for 1 h. The cells were then stained with propidium iodide and fluorescein isothiocyanate-labeled Annexin V at 4 °C for 10 min in the dark. Then apoptosis levels were measured using a FACSCaliber (BD Biosciences, San Jose, CA, USA).

**Statistical analysis**

Data are presented as mean ± standard deviation (SD). The statistical significance of the differences between groups was evaluated by Student’s t-test, and a value of p < 0.05 was considered significant.

**RESULTS**

**MFAP2 was expressed in lung cancer cells**

To identify the role of MFAP2 in lung cancer progression, its expression was evaluated in human lung cancer tissue samples and cells. Using the TCGA database, MFAP2 was abnormally highly expressed in human lung cancer (Figure 1 A). Furthermore, qPCR assays were conducted to detect the mRNA levels of MFAP2 in the BEAS-2B lung epithelial cell line and in four types of lung cancer cell lines (A549, PC-9, NCI-H520, and NCI-H1703). The results indicated that the mRNA for MFAP2 was upregulated in lung cancer cells (Figure 1 B). The A549 and NCI-H1703 cells showed higher mRNA levels of MFAP2, when compared to other lung cancer cell lines (Figure 1B). Immunoblot assays confirmed the expression of MFAP2 in these cell lines and showed high MFAP2 expression levels in the four types of lung cancer cells (Figure 1 C). Together, the results showed that MFAP2 was highly expressed in lung cancer cells.

**MFAP2 ablation suppressed the viability of lung cancer cells and stimulated apoptosis.**

Because MFAP2 was highly expressed in human lung cancer cells, its role in lung cancer was determined. MFAP2 siRNA was transfected into A549 and NCI-H1703 cells. The transfection of MFAP2 siRNA significantly decreased its expressions in A549 and NCI-H1703 cells at the mRNA and protein levels (Figure 2 A and B). Knockdown of MFAP2 decreased the optical density at 450 nm in A549 and NCI-1703 cells, suggesting inhibition of cell viability (Figure 2 C). Similarly, depletion of MFAP2 decreased the colony numbers in A549 and NCI-1703 cells (Figure 2 D). Flow cytometry showed that ablation of MFAP2 stimulated apoptosis in A549 and NCI-1703 cells, with increased numbers of apoptotic cells (Figure 2 E). Together, the results showed that MFAP2 depletion suppressed the viability of lung cancer cells and stimulated cell apoptosis.
after transfection of control or MFAP2 siRNA. (C) The CCK-8 assays determining the optical density values at 450 nm of A549 and NCI-1703 cells after transfection of control or MFAP2 siRNA. (D) Colony formation assays showing the colony numbers of A549 and NCI-1703 cells after transfection with control or MFAP2 siRNA. (E) Flow cytometry assays showing the apoptosis percentages of A549 and NCI-1703 cells after transfection with control or MFAP2 siRNA. Data are presented as the mean ± SD. MFAP2 siRNA group vs. the control siRNA group, **p < 0.01, ***p < 0.001.

MFAP2 knockdown inhibited migration and invasion of lung cancer cells.

The effects of MFAP2 on the motility of lung cancer cells was determined. Its depletion increased the wound width after 24 h in A54 and NCI-1703 cells (Figures 3A and 3B). MFAP2 knockdown suppressed lung cancer cell migration and suppressed the invasion of A549 and NCI-1703 cells, with decreased numbers of invasive cells (Figure 3C, 3D). MFAP2 knockdown therefore inhibited the migration and invasion of lung cancer cells.

Figure 3: MFAP2 knockdown inhibited the migration and invasion of lung cancer cells. (A, B) Wound closure assays showing the wound healing of A549 and NCI-1703 cells after transfection with control or MFAP2 siRNA. Representative images are shown in (A). The wound width is shown in (B). (C, D) Transwell assays showing the invasion of A549 and NCI-1703 cells after transfection with control or MFAP2 siRNA. Representative images are shown in (C). The invasive cell number is listed in (D). Data are presented as the mean ± SD. MFAP2 siRNA group vs. the control siRNA group; *p < 0.05, **p < 0.01

Depletion of MFAP2 suppressed EMT in lung cancer cells

MFAP2 knockdown increased the mRNA levels of E-cadherin and downregulated N-cadherin, snail, MMP-2, and MMP-9 in A549 and NCI-1703 cells (Figure 4), showing that depletion of MFAP2 suppressed the EMT in lung cancer cells.

Figure 4: Depletion of MFAP2 suppressed the EMT process in lung cancer cells. Immunoblot assays showing the expressions of E-cadherin, N-cadherin, Snail, MMP-2, and MMP-9 in A549 and NCI-1703 cells after the transfection with control or MFAP2 siRNA. Data are presented as the mean ± SD; *p < 0.05, **p < 0.01

MFAP2 mediated Wnt/β-catenin pathway in lung cancer cells

Immunoblot assays showed that the depletion of MFAP2 decreased the phosphorylation levels of GSK-3β and the expression levels of β-catenin in both A549 and NCI-1703 cells (Figure 5), showing that MFAP2 mediated the Wnt/β-catenin axis in lung cancer cells.

Figure 5: MFAP2 mediated the Wnt/β-catenin pathway in lung cancer cells. Immunoblot assays showing the expression of phosphorylated GSK-3β and the expression of GSK-3β and β-catenin in A549 and NCI-1703 cells after transfection with control or MFAP2 siRNA. Data are presented as the mean ± SD; ***p < 0.001

DISCUSSION

The incidence and mortality of lung cancer in men ranks first among all malignancies [2]. However, the pathogenesis of lung cancer...
remains unclear. Early lung cancer lacks obvious symptoms, and when patients seek treatment, they are often in advanced stages, and conventional treatment methods, including surgical resection, radiotherapy, and chemotherapy, are ineffective, which is also the main reason for the poor prognoses of lung cancer patients [14]. Targeted therapy for lung cancer has made some progress, and a variety of targeted therapy drugs for lung cancer have been clinically used to improve the prognoses of patients [5]. However, to further improve the survival of lung cancer patients, it is important to further characterize the pathology of lung cancer and develop new and more effective targets. In this study, a microfibrillar-associated protein, MFAP2, was found to be highly expressed in lung cancer cells, where it affected multiple cellular processes. Therefore, MFAP2 plays a vital role in lung cancer progression.

MFAP2 affected the viability as well as apoptosis of lung cancer cells. Using wound healing, Transwell, and immunoblot assays, the effects of MFAP2 on the motility and EMT of lung cancer cells were determined. The results showed that MFAP2 plays an important role in lung cancer, consistent with the results of previous studies [9,12,15].

MFAP2 has been correlated with angiogenesis and prognosis in HCC [16]. In addition, MFAP2 is a diagnostic and prognostic biomarker of papillary thyroid cancer (PTC) [9] and can potentiate the motility of melanomas [12]. Importantly, MFAP2 promotes the motility of lung cancer cells. MFAP2 also contributes to the EMT in gastric cancer cells [10]. Another study reported that MFAP2 was highly expressed in gastric cancers and contributed to cell motility [15]. These studies confirmed that MFAP2 had extensive and complex regulatory effects on different types of tumors. Because MFAP2 mediates the Wnt/β-catenin axis in lung cancer cells, its mechanism of action still needs further study.

The Wnt signaling pathway promotes β-catenin accumulation in cells, and the abnormal expression of β-catenin can induce the occurrence of tumors [17]. This pathway mediates the proliferation, motility, apoptosis, and EMT of several types of tumors [18]. In the present study, MFAP2 affected the viability and motility of lung cancer cells via the Wnt pathway, suggesting that the Wnt/β-catenin pathway may serve as a promising target in lung cancer.

Taken together, MFAP2 was highly expressed in lung cancer cells. MFAP2 promoted the viability, motility, and EMT in lung cancer cells and suppressed apoptosis by mediating the Wnt/β-catenin pathway.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiuyun He and Yunfeng Qiao designed and conducted the experiments. Xiuyun He analyzed and interpreted the data. Yunfeng Qiao wrote the manuscript, with contributions from all co-authors.

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