Introduction

Lung cancer, which is generally divided into non-small cell lung cancer (NSCLC, about 85% cases) and small cell lung cancer (SCLC, about 15% cases), is the leading cause of cancer-related mortality worldwide (1). The major histological subtypes of NSCLC are adenocarcinoma and squamous cell carcinoma (2). When diagnosed at an earlier stage (stage I), surgical therapy of NSCLC possesses a good prognosis for localized and small tumor patients with a 5-year survival rate of 70–90% (3). Unfortunately, most patients (75%) are diagnosed at later stages (4) and although prominent progress in the therapy of advanced stage lung cancer (stage III/IV) has been made in recent years, the survival rate remains poor. Data from the UK Office of National Statistics revealed that the one-year survival rate for stage IV patients is only 15–19% (5).

Chemotherapy is a central strategy for both surgical and inoperable cancer patients, while platinum is mild in...
the treatment of advanced NSCLC (6). Drug resistance and adverse drug reactions, however, greatly weaken the therapeutic effects of these strategies. In recent years, the therapeutic strategy for advanced NSCLC has transformed from histopathology-based traditional chemotherapy into carcinogenic factor-based individualized precision treatment (7). Previous study revealed that MTA1 might be an important biomarker in the diagnosis of NSCLC (8). Hyperactivation of Wnt/β-catenin and transforming growth factor-β (TGF-β) signaling has been implicated in the metastasis of NSCLC (9). Other studies showed that phosphatase and tensin homolog (PTEN) deficiency (10) and over-expression of insulin-like growth factor-1R (IGF-1R) (11) were closely associated with poor prognosis in NSCLC patients. Although therapeutic targets and biomarkers have, to some degree, contributed to improving NSCLC diagnosis and therapy, the complex pathogenesis of NSCLC remains a challenge and thus necessitates additional genetic information to develop better medical treatment (12).

FAM46 proteins, which belong to the nucleotidyltransferase (NTase) fold superfamily (13) of proteins, contain four human paralogs (FAM46A, FAM46B, FAM46C, FAM46D). FAM46A has been reported as a positional candidate for human retinal diseases and might associate with NSCLC (14,15). FAM46B is described as a potential marker for refractory lupus nephritis (16), while FAM46C is considered as a type I interferon-stimulated gene (ISG), which promotes replication of certain viruses (17). Results from gene set enrichment analysis (GSEA) revealed that lower FAM46B expression is correlated with the metastasis of lung cancer and involves Wnt signaling pathway (Supplementary file). However, only a few reports focus on the relationship between FAM46B and NSCLC. Hence, the present study examined the expression of FAM46B in 5 NSCLC cell lines, investigated the effects of FAM46B on NSCLC invasiveness, and probed the involvement of downstream β-catenin signaling in this process.

Methods

Chemicals and reagents

The cell counting kit-8 (CCK-8) assay kit was obtained from Signal way& Antibody (SAB, MA, USA). Trizol reagent and reverse transcription kits were purchased from Thermo Fisher (MA, USA). Crystal violet was obtained from Solarbio (Shanghai, China). FAM46B and vascular endothelial growth factor (VEGF) antibodies were purchased from Proteintech (IL, USA). β-catenin and matrix metalloproteinase 7 (MMP7) antibodies were obtained from Abcam Biotech (Cambridge, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Cell Signaling Technology (MA, USA).

Patients

Human cancer tissues and corresponding paracancerous normal tissues were collected from lung cancer patients [37 patients with a mean ± standard error of mean (SEM) age of 55±10 years] who received pulmonary resection. This study was conducted with approval from the Ethics Committee of our hospital (YS-2019-065) and in accordance with the Declaration of Helsinki. Written informed consent was obtained from patients. Relative mRNA expression of FAM46B and protein levels of FAM46B and β-catenin were detected in the paired tissues.

Cells

NSCLC cell lines (A549, H1299, H1975, H292 and H358) and human bronchial epithelial cells (16HBE) were obtained from cell bank of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured with Dulbecco’s modified Eagle medium (DMEM) medium (HyClone, Logan, UT, USA) supplemented with 100 U/mL penicillin and 10% fetal bovine serum (FBS) (Gibco Company, USA) at 37 °C (5% CO2). Cells at logarithmic phase were used for follow-up experiments.

Construction of lentiviral vector

FAM46B (NM_052943.3) shRNA and over-expression lentiviral vectors were designed (shown in Table 1), constructed and verified by JRDun Biotechnology (Shanghai, China). Viral supernatants were diluted in the medium at appropriate concentrations and added to monolayer cells in follow-up experiments.

Cell proliferation, migration and invasion

Viral supernatants were diluted in the medium at appropriate concentrations and added to monolayer cells. Cells were then cultured with DMEM medium supplemented with 10% FBS at 37 °C (5% CO2) for 48 h.
Cells were harvested at 0, 24 and 48 h after treatment. Cell proliferation was assessed using CCK-8 assay kit following manufacturer’s instructions.

For transwell migration assay, a transwell chamber (corning-costar) was used according to manufacturer’s instructions. Briefly, cell culture inserts (aperture 6.5 mm) were rehydrated with serum-free DMEM at 37 °C for 2 h, and then 1×10^5/mL cells (in 500 μL serum-free DMEM) were seeded onto the upper chamber with 0.1% dimethyl sulfoxide (DMSO) control or viral supernatants/test drug. Meanwhile, 750 μL DMEM with 10% FBS was added into the lower chamber. After 24 h, cells on the upper surface of the membrane were wiped off by gentle swabbing. The cells that had migrated through the pores were fixed. After standard staining and microscopic examination, five random images per chamber were obtained at 200× magnification. Relative migration was calculated by counting cells and normalized to the negative control cells.

Invasion of cells into matrigel was determined similar to the migration assay. Briefly, after pre-hydration, 80 μL matrigel was added into the invasion chambers following incubation at 37 °C for 30 min. 2×10^5/mL cells (in 500 μL serum-free DMEM) were seeded onto the upper chamber with 0.1% DMSO control or viral supernatants/test drug. Meanwhile, 750 μL DMEM with 10% FBS was added into the lower chamber. After 24 h, cells on the upper surface of the membrane were wiped off by gentle swabbing. The cells that had migrated through the pores were fixed. After standard staining and microscopic examination, five random images per chamber were obtained at 200× magnification. Relative invasion was calculated by counting cells and normalized to the negative control cells.

Quantitative real-time polymerase chain reaction (PCR)

Total mRNA (2 μg) of cultured NSCLC cells were extracted using Trizol reagent, quantified by the Qubit®2.0 quantitative machine using Qubit RNA HS Assay Kit (Life, MA, USA), and then reverse transcribed into cDNA using the first-strand cDNA synthesis kit. Then, reverse transcription-PCR (RT-PCR) analysis was performed on a quantitative reverse transcription-PCR (qRT-PCR) machine (ABI-7500, USA) using SYBR Green reagents and the following primers: pri-FAM46B-sense (S) 5′-ACAAGACGGAGGAGCUCAAUU, pri-FAM46B-antisense (AS) 5′-CAGACATGGGAGTGGACGAG-3′, pri-GAPDH-sense (S) 5′-AATCCCATCACCATCTTC, pri-GAPDH-antisense (AS) 5′-AGGCTGTTGTGCACATCTTC. Data were calculated by the 2−ΔΔCq method (18). GAPDH was used as a reference gene.

Western blot analysis

The total protein of each cell sample was isolated and detected by a bichinonic acid (BCA) protein assay kit following manufacturer’s instructions. Protein (35 μg) was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk at 25 °C for 1 h. Subsequently, relative membranes were incubated with the following primary antibodies: FAM46B (1:1,000), β-catenin (1:5,000), MMP7 (1:1,000), VEGF (1:1,000), and GAPDH (1:1,000). Membranes were then washed with phosphate buffered saline (PBS) and subsequently incubated with horseradish peroxidase-conjugated (HRP-conjugated) goat anti-rabbit IgG secondary antibody (Beyotime Biotech, Shanghai, China) for 1 h. Finally, protein bands were detected by an enhanced chemiluminescence (ECL) detection kit (Beyotime Biotech, Shanghai, China). Blots against

| Description | Sequences or primers (5′-3′) |
|-------------|-----------------------------|
| FM46B (NM_052943.3) shRNA | Forward: GCAAGACGGAGGAGCUCAAUU, Group 1-reversed: UUGAGCUCCAGGUCUUGCUU |
| | (762–780) |
| FM46B (NM_052943.3) OE | Forward: CGGAATTCTATGATGCGTGGAGAGG, Reversed: CGGGATCTCTAGTTACAAGGCAGGCAGGG |
| | (173–1450) |

OE, over-expression.
GAPDH served as loading controls.

**Statistics analysis**

The data were presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 19.0 software (SPSS, Inc., Chicago, USA). Analysis of variance (ANOVA) and paired Student's t-test, along with the Dunnett's test and post-hoc tests least significant difference test, were used for comparison between groups. P<0.05 was considered as statistically significant.

**Results**

**Differentially expressed FAM46B and β-catenin in paracancerous and cancer tissues of lung cancer patients**

The present results revealed that relative mRNA expression of FAM46B was lower in lung cancer tissues compared to paired paracancerous tissues (Figure 1). Likewise, the protein level of FAM46B displayed a similar trend as its mRNA expression. In contrast, the protein level of β-catenin was higher in lung cancer tissues compared to paired paracancerous tissues.

**Screening of cell lines A549 and H1975**

Results from GSEA revealed a correlation between lung cancer metastasis and reduced FAM46B expression (Supplementary file). In the present study, 5 NSCLC cell lines (A549, H1299, H1975, H292 and H358) and 1 human bronchial epithelial cell line (16HBE) were used to investigate the mRNA and protein levels of FAM46B. The results indicated that FAM46B displayed lower expressions in A549 and H292 cells but higher expressions in H1975 cells compared with 16HBE cells (Figure 2). These three cell lines (A549, H292 and H1975) were subsequently used in follow-up experiments.

**Effects of FAM46B over-expression on A549 and H292 cells**

Our earlier findings indicated that lower FAM46B expression was associated with the metastasis of lung cancer and suggested a molecular mechanism involving Wnt signaling (Supplementary file). In the present study, we investigated the effects of FAM46B over-expression on the
proliferation, migration and invasion of H292 and A549 cells, as well as on β-catenin, MMP7 and VEGF protein levels. As shown in Figure 3, proliferation, migration and invasion of H292 and A549 cells were suppressed by FAM46B over-expression. Moreover, the protein levels of β-catenin, MMP7 and VEGF were decreased by FAM46B over-expression both in A549 and H292 cells.

**Discussion**

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Air contamination and cigarette smoke are primary causes of lung cancer (20,21). Traditional strategies for lung cancer treatment frequently lead to toxic side effects and are unavailable for some patients (22). Compared with histopathology-based traditional chemotherapy, molecular-targeted therapy shows reduced toxicity to normal tissues, high selectivity and better promise for eradicating the tumor (7,23,24). Our present study suggested that FAM46B acts as a tumor suppressor in NSCLC. Its expression is markedly reduced in lung cancer tissues compared to paired paracancerous tissues. Furthermore, over-expression of FAM46B weakened the proliferation, migration and invasion of H292 and A549 cells, while inhibiting β-catenin/MMP7 signaling. β-catenin is the major component of Wnt signaling and has been shown to influence epithelial mesenchymal transition (EMT) (25,26). Hyperactivation of β-catenin is known to enhance cell migration and invasion, as well as induce EMT in NSCLC (27). MMP7 is a downstream target of β-catenin and involved in tumor progression by promoting EMT, cell invasion and metastasis (28). A previous report indicated that phospholipase Cδ1 suppresses cell migration and invasion of breast cancer cells via down-regulation of β-catenin/MMP7 signaling pathway (29). VEGF is known as a key regulatory factor involved in the regulation of tumor growth by activating host vascular endothelial cells, stimulating tumor angiogenesis, and enhancing malignant tumor progression (30). The present study revealed that FAM46B over-expression significantly reduced the protein levels of β-catenin, MMP7 and VEGF in both A549 and H292 cells. Conversely, FAM46B knockdown elevated the protein levels of β-catenin, MMP7 and VEGF, and stimulated proliferation, migration and invasion of H1975 cells. These effects were weakened by
Figure 3 Proliferation (A), migration (B) and invasion (C) of A549 and H292 cells were suppressed by FAM46B over-expression; (D) proteins level of β-catenin, MMP7 and VEGF were decreased by FAM46B over-expression both in A549 and H292 cells. **, P<0.01, compared with EPC group (n=3). EPC, empty plasmid control; OE, over-expression; MMP7, matrix metalloproteinase 7; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Figure 4 Proliferation (A), migration and invasion (B) of H1975 cells were enhanced by FAM46B shRNA knockdown but weakened by co-treatment with XAV939; (C) Protein levels of β-catenin, MMP7 and VEGF were increased by FAM46B shRNA knockdown but decreased by co-treatment with XAV939. *, P<0.05, **, P<0.01, compared with EPC group. #, P<0.05, ##, P<0.01, data in XAV939 + FAM46B shRNA/shRNA group compared with FAM46B shRNA group. $, P<0.05, $$, P<0.01, data in XAV939 + FAM46B shRNA/shRNA group compared with XAV939 + EPC group (n=3). EPC, empty plasmid control; OE, over-expression; MMP7, matrix metalloproteinase 7; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
co-treatment with the Wnt/β-catenin signaling inhibitor XAV939, suggesting that β-catenin signaling is likely to mediate the functions of FAM46B in NSCLC.

Taken together, our study demonstrated that proliferation, migration and invasion of NSCLC cells are inhibited by FAM46B over-expression and stimulated by FAM46B knockdown. Hence, FAM46B may function as a tumor suppressor in NSCLC by regulating β-catenin, MMP7 and VEGF.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.07.27). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethics Committee of the Sixth People’s Hospital affiliated to Shanghai University of Medicine & Health Sciences and conducted in accordance with the Declaration of Helsinki 2013 (YS-2019-065). Signed written informed consents were obtained from the participants and/or guardians.

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Identification of relationship between FAM46B and the metastasis of lung cancer, processes by Gene Set Enrichment Analysis (GSEA). GSEA identified significant negative association between FAM46B and the metastasis of lung cancer.

Dataset
Lung cancer TCGA.FAM46B_profile_in_Lung_cancer_TCGA.cls#FAM46B

Phenotype
FAM46B_profile_in_Lung_cancer_TCGA.cls#FAM46B

Upregulated in class
FAM46B_neg

Gene set
GRADE_METASTASIS_DN

ES
-0.536809

NES
-2.1690097

Nominal P value
0.0

FDR Q value
1.8023093E–4

FWER P value
0.002

ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate; FWER, familywise error rate.
GSEA identified significant negative association between FAM46B and Wnt signaling pathway in The Cancer Genome Atlas (TCGA) lung cancer dataset.

Dataset
Lung cancer TCGA.FAM46B_profile_in_Lung_cancer_TCGA.cls#FAM46B

Phenotype
FAM46B_profile_in_Lung_cancer_TCGA.cls#FAM46B

Upregulated in class
FAM46B_neg

Gene set
REACTOME_SIGNALING_BY_WNT

ES
-0.47817597

NES
-2.014252

Nominal P value
0.0

FDR Q value
6.2330585E–4

FWER P value
0.011

ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate; FWER, familywise error rate.