

# FHL1 Overexpression as A Inhibitor of Lung Cancer Cell Invasion via Increasing *RhoGDIβ* mRNA Expression

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## Abstract

**Objective:** Four and a half LIM-1, LIM-2, LIM-3 (LIM) protein 1 (FHL1) is one of the FHL protein family, which is regarded as a tumor suppressor in the multiple malignant tumors. In this study, we aimed to explore the regulatory effects and mechanisms of FHL1 on lung cancer cell invasion.

**Materials and Methods:** In this experimental study, bioinformatics analysis of *FHL1* transcripts in human lung adenocarcinomas of TCGA database was performed. Quantitative real-time polymerase chain reaction (PCR) was performed to detect *FHL1* mRNA expression in 15 paired human lung cancer tissues and their adjacent normal lung tissues, or lung cancer cell lines (A549 and H1299) in comparison with human bronchial epithelial cell line (Beas-2B). Moreover, western blot was used to analyze FHL1 and rho GDP-dissociation inhibitor beta (*RhoGDIβ*) protein expression in the indicated cell lines. Also, transwell assays were employed to measure the migrated, and invaded of indicated cell lines.

**Results:** *FHL1* transcripts were downregulated in the human lung adenocarcinoma. The impaired *FHL1* transcripts were positively correlated with advanced tumor node metastasis (TNM) stage. Moreover, as compared to the adjacent normal lung tissues, *FHL1* mRNA was low expressed in 15 paired human lung cancer tissues than their adjacent normal lung tissues. Besides, *FHL1* mRNA and protein expression were also reduced in H1299 and A549 cell lines in comparison with Beas-2B cell line. Overexpressed FHL1 protein inhibited the invasive ability of H1299 and A549 cell lines. Mechanically, FHL1 protein overexpression increased the *RhoGDIβ* protein and mRNA abundance, while knockdown of *RhoGDIβ* protein, completely restored the invasion ability of A549 (Flag-FHL1) cell line.

**Conclusion:** Our findings indicated that as a key FHL1 downstream regulator, *RhoGDIβ* is in charge of FHL1 inhibiting lung cancer cell invasion abilities, providing a critical insight into understanding the role of FHL1 for lung cancer development.

**Keywords:** FHL1, Invasion, Gene Expression, Lung Cancer, rho GDP-Dissociation Inhibitor Beta

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## Introduction

Lung cancer, one of the most common cancers, has received a lot of attention. While its morbidity and mortality are increasing year by year, the poor 5-year survival for non-small-cell lung cancer is merely about 15% (1, 2). Furthermore, based on traditional pathological and clinical parameters, non-small-cell lung cancer outcomes could not be determined (3). Due to the poor understanding of lung cancer mechanisms, tumorigenesis and progression, the advances of effective treatments remain limited. Therefore, development of novel molecular targets, biomarkers and novel therapeutic strategies are necessary.

Four and a half LIM-1, LIM-2, LIM-3 (LIM) protein 1 (FHL1) belongs to the FHL protein family, which comprises of four LIM domains and an N-terminal half LIM domain (4, 5). Multiple investigations on clinical samples have revealed that FHL1 protein expression inhibition in the several types of tumors, including lung (6), liver (7), breast (8), gastric (9), and prostate

cancer (10). Researches on human totally lung cancer patients who received radiotherapy have indicated that the downregulation of FHL1 protein has resulted in significantly lower disease-free survival (6). Recently, They reported the inhibitory effects of FHL1 protein on lung cancer cell growth. FHL1 protein overexpression induced G1 and G2/M cell cycle arrest through inhibiting protein expression of Cyclin A, Cyclin B1 and Cyclin D, as well as inducing the expression of p21 and p27 protein, suggesting the tumor suppressor effect of FHL1 on human lung cancer cell growth. Moreover, SRC protein promoted the phosphorylation of FHL1 protein, then increased the directly binding with BCLAF1 protein in the nucleus, and finally promoted tumor cell growth (11), revealing that the role of FHL1 and its mechanism in the cancer progression is complicated.

*RhoGDIβ* protein belongs to the family of RHO guanosine diphosphate dissociation inhibitors (12, 13). Rho GTPases widely participate in a number of cellular responses, particularly in the cell motility (14).

Several investigations have indicated that RhoGDI $\beta$  is an aggressive human cancer marker (15). The protein and mRNA expression of RhoGDI $\beta$  was reported to be downregulated in both adenocarcinoma and squamous lung cell carcinoma (16). A reduction of tumor versican was observed upon overexpression of RhoGDI $\beta$  protein, and thereby suppressed lung metastasis *in vivo* mouse models (17). However, other researchers have revealed the oncogenic function of RhoGDI $\beta$ . For instance, RhoGDI $\beta$  has been shown to mediate ATG7-induced bladder cancer invasion (18). RhoGDI $\beta$  prevented the lung colonization of bladder cancer through unexpected targeting RhoC protein and reducing the activation of RhoC (19).

Based on the above, the therapeutic targeting of these FHL1/RhoGDI $\beta$  may appear to be a promising anti-cancer strategy. Therefore, we intended to figure out the relationship between FHL1 protein expression and human lung cancer cell invasion, and study the mechanism that involved in this progress, especially the function of RhoGDI $\beta$ .

## Materials and Methods

### Ethical considerations

This study was permitted by the Ethics Committee of Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School (2020-103-27). Written informed consents were obtained from all participants.

### Plasmids, reagents and antibodies

In this study, shRNA specific targeting RhoGDI $\beta$  (shRhoGDI $\beta$ ) were obtained from BioVector NTCC Inc. (Cat No. 58897, Shanghai, China). The PCR-amplified FHL1 fragment was inserted into the pcDNA3.1 vector (Cat No. P8990, Miaolinbio, China) to construct the Flag-tagged FHL1 plasmid. Before the transfection, the plasmid preparation kit (Cat No. D0003, Beyotime, China) was used to pretreating the plasmids. The TRIzol reagent (Cat No.15596026) and SuperScript<sup>TM</sup> First-Strand Synthesis system (Cat No. 11904018) were acquired from Invitrogen (China). The antibodies specific against FHL1 (Cat No. ab255828), Flag (Cat No. ab205606), and GAPDH (Cat No. ab9485) were purchased from Abcam (China). Antibodies against RhoGDI $\alpha$  (Cat No. sc-373724), RhoGDI $\beta$  (Cat No. sc-271108) and  $\beta$ -Actin (Cat No. sc-8432), were purchased from Santa Cruz (China).

### Cell culture and transfection

Human lung cancer cell line A549 (BFN60800665, BLUEFBIO<sup>TM</sup>, China), H1299 (BFN60804058, BLUEFBIO<sup>TM</sup>, China) and human bronchial epithelial cell line Beas-2B (BFN608009328, BLUEFBIO<sup>TM</sup>, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Cat No. C11995500BT, Gibco, China), supplemented with 2  $\mu$ M of L-glutamine (Cat

No. 25030149, Gibco, China), 25  $\mu$ g/ml of gentamycin (Cat No. 15710-049, Thermo Fisher Scientific, China), and 10% heat-inactivated fetal bovine serum (FBS, Cat No. 10099-141C, Gibco, China). Cell transfections were performed by using PolyJet<sup>TM</sup> DNA In Vitro Transfection Reagent (Cat No. SL100468, SignaGen Laboratories, USA), according to the manufacturer's instruction and described in the previous studies (18). For the transfection of pcDNA3.1/Flag-FHL1 into A549 and H1299 cell lines, 2  $\mu$ g of plasmids were used, and the stable transfectants were generated by G418 selection (500 g/ml). For the transfection of shRhoGDI $\beta$  into A549 (Flag-FHL1), 2  $\mu$ g of plasmids were used, and the stable transfectants were generated by puromycin selection (2  $\mu$ g/ml).

### Western blot analysis

Beas-2B, A549 and H-1299 cell lines and their transfectants were cultured at 37°C in 5% CO<sub>2</sub> and 10% FBS medium for 12 hours till to 70-80% concentration. After 12 hours for culturing cells in 0.1% FBS medium, the 10% FBS DMEM medium was used for another 12 hours. Afterward, whole cell extracts were prepared with the cell lysis buffer [10 mM Tris-HCl (Cat No. T1090), pH=7.4, 1% sodium dodecyl sulfate (SDS, Cat No. S8010), and 1 mM Na<sub>3</sub>VO<sub>4</sub> (Cat No. IS0210)] (all of them from the Solarbio Life Science, China) and then were subjected to Western Blot analysis according to the previous study (20).

### Quantitative real-time polymerase chain reaction

A Fast SYBR Green Master Mix kit (Cat No. 4385614, Applied Biosystems, China) was used to detect the mRNA expression. The primers were:

human *FHL1*-

F: 5'-CTG CTG CCT GAA A-3'  
R: 5'-TCT CCT GCC ACA AT-3'

human *RhoGDI $\beta$* -

F: 5'-ACC CGG CTC ACC CTG GTT TGT-3'  
R: 5'-ACC CCA GTC CTG TAG GTG TGC TG-3'

human  $\beta$ -*Actin*-

F: 5'-CTC CAT CCT GGC CTC GCT GT-3'  
R: 5'-GCT GTC ACC TTC ACC GTT CC-3'

### Cell migration and invasion assay

For migration assays, three transwell chambers (Cat No. 353097, Corning, USA) were used for each individual cell group. The invasion kit (Cat No. 354480, BD Biosciences, USA) was used for each individual cell group. The stable transfectants which were under selected for 3-4 weeks with the indicated antibiotics, puromycin (Cat No. A1113803, Gibco, China), or G418 (Cat No. 10131027, Gibco, China). And then the stable transfectants were used to do the cell migration and

invasion assays and the normalized invasion rate was calculated according to the manufactures' instruments. According to the previous study (21), six photographs of each chamber were taken using the microscope, Olympus DP71 [Model No. DP71, Olympus (China) Co., Ltd, China], and the number of the migrated or invaded cells was counted using the "Image J" software. Based on the number of migrated or invaded cells, the migration rate was normalized with the nonsense control cells, while the invasion rate was firstly calculated by dividing the number of migrated cells, and then were normalized with nonsense control cells (18). The presented data are representative of three independent experiments.

### Human lung cancer tissue specimens

All human lung cancer tissue specimens (15 pairs of human lung cancer tissues and their paired adjacent normal lung tissues) were obtained from patients who received surgery at the Affiliated Hospital of Nanjing University Medical School (Nanjing, China) during 2020-2021.

### Bioinformatics analysis of TCGA database

Because of the aberrant silencing attribution of FHL1 in human cancers, the bioinformatics analysis was initially performed on *FHL1* transcripts with 574 lung cancer patients from TCGA database. UALCAN (<http://ualcan.path.uab.edu>) (22) was used to perform the bioinformatics analysis of *FHL1* transcripts in human lung cancer tissues.

### Statistical analysis

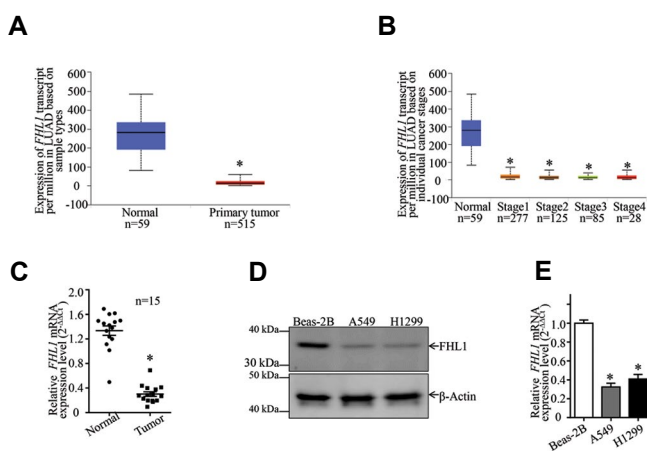
GraphPad Prism 6.0 Software (GraphPad Software, USA) was employed for statistical analysis. All data demonstrated mean  $\pm$  SD of triplicate assays. Student's t test was used to detect the significance of differences between groups. One-way ANOVA test was performed to detect the significant differences of multiple comparisons. The differences were considered significant at  $P < 0.05$ .

## Results

### FHL1 protein expression was inhibited in the human lung cancer tissues and cell lines

The bioinformatics results revealed that the *FHL1* transcripts were downregulated in the human lung cancer tissues when compared with the normal tissue samples (Fig.1A). Furthermore, the impaired *FHL1* transcripts were positively correlated with advanced TNM stage (Fig.1B). Analysis of the *FHL1* mRNA expression showed a downregulation in the human lung cancers (Fig.1C). Next, the protein and mRNA expression of *FHL1* was examined among Beas-2B, A549 and H1299 cell lines. The result showed that *FHL1* mRNA and protein expression were decreased in the H1299 and A549 cell lines in comparison with Beas-2B cell lines (Fig.1D, E). Our data showed that

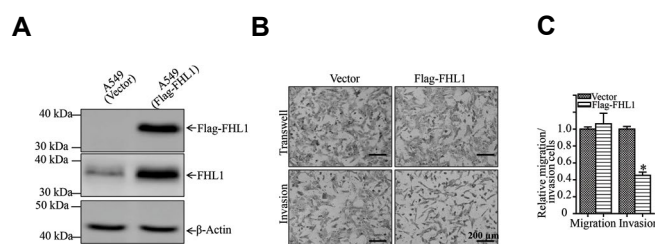
*FHL1* mRNA and protein expression were inhibited in human lung cancers.



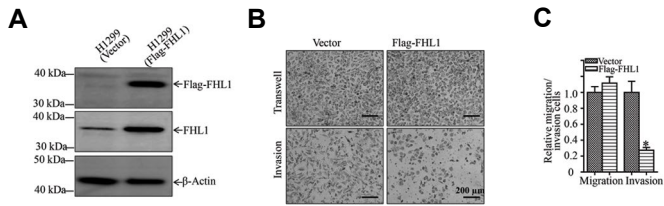
**Fig.1:** FHL1 was downregulated in human lung cancer tissues and cell lines. **A, B.** Bioinformatics analysis was performed on the *FHL1* transcript with 574 lung adenocarcinoma (LUAD) patients from the TCGA database. The *FHL1* transcripts were reduced in lung cancer tissues, in comparison with the normal lung tissues. The expression of *FHL1* transcript was downregulated in lung cancer patients with an advanced stage. **C.** *FHL1* mRNA expression was detected in 15 paired lung cancer tissues and their adjacent normal tissues. Student's t test was used to detect the P value,  $P < 0.05$ . **D.** Expression of FHL1 in Beas-2B, A549, and H1299 cell lines was determined by western blot assay,  $\beta$ -Actin was used as a protein loading control. **E.** *FHL1* mRNA expression was determined by real-time polymerase chain reaction (PCR), and the asterisk (\*) represents a notable decrease from normal Beas-2B cells ( $P < 0.05$ ).

### FHL1 inhibition was essential for human lung cancer cell invasion

In order to investigate the relevance between FHL1 protein and human lung cancer development, the Flag-tagged FHL1 overexpression plasmid was stably transfected into A549 cell line (Fig.2A). Furthermore, the result revealed that FHL1 protein overexpression suppressed the invasion of these cells (Fig.2B, C). We stably transfected Flag-FHL1 into H1299 cell line (Fig.3A), and also, found that FHL1 overexpression inhibited the invasion of H1299 cell line (Fig.3B, C). Our results showed a new negative regulatory effect of FHL1 on human lung cancer invasion.



**Fig.2:** FHL1 overexpression inhibited the invasion of A549 cell line. **A.** The Flag-tagged FHL1 plasmid was stably transfected into A549 cell line. **B.** The invasive ability was determined using the Biocoat™ matrigel® invasion chamber, while the migration ability was detected using the same system without the matrigel (scale bar: 200  $\mu$ m). **C.** The invasive ability was normalized to the insert control. The asterisk (\*) represents a significant reduction as compared to A549 (Vector) cell lines ( $P < 0.05$ ).

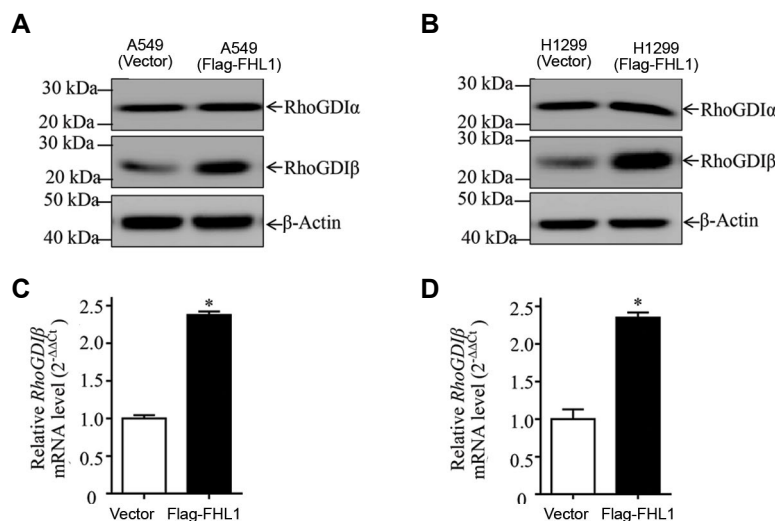


**Fig.3:** FHL1 overexpression inhibited the invasion of H1299 cell line. **A.** The Flag-tagged FHL1 plasmid was stably transfected into the H1299 cell line. **B.** The invasive ability was determined using the Biocoat™ matrigel® invasion chamber, while the migration ability was detected using the same system without the matrigel (scale bar: 200 μm). **C.** The invasive ability was normalized to the insert control. The asterisk (\*) represents a significant reduction as compared to H1299 (Vector) cell lines (P<0.05).

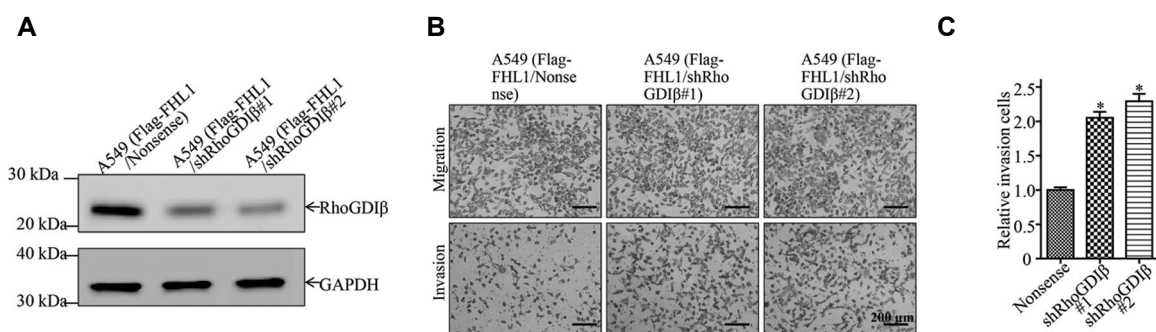
**FHL1 protein suppression of lung cancer invasion was regulated by decreasing *RhoGDIβ* mRNA expression**

In order to investigate the mechanism of FHL1 protein in regulating lung cancer invasion, western blot was carried out to select the potential FHL1 downstream effectors. The results showed that the overexpression of FHL1 only increased *RhoGDIβ* protein abundance, and had no remarkable effect on *RhoGDIα* protein expression in both A549 and H1299 cell

lines (Fig.4A, B), indicating that FHL1 overexpression exerts a promotion effect on the *RhoGDIβ* protein expression in human lung cancer cells. Additionally, in order to investigate the mechanism underlying the FHL1 upregulating *RhoGDIβ* protein, we firstly detected a *RhoGDIβ* mRNA abundance. As shown in Figure 4C, D, the *RhoGDIβ* mRNA level was significant increased in the FHL1 overexpression transfectants. Therefore, it was anticipated that *RhoGDIβ* might be responsible for the FHL1 inhibition in human lung cancer cell invasion. Following, sh*RhoGDIβ*#1 and sh*RhoGDIβ*#2 were stably transfected into A549 (Flag-FHL1) cells (Fig.5A). Subsequently, invasion assay was performed and the data revealed that *RhoGDIβ* knockdown enhanced the invasion ability of A549 (Flag-FHL1) cells, in comparison to those observed in their scramble nonsense transfectants A549 (Flag-FHL1/Nonsense) cells (Fig.5B, C), demonstrating that *RhoGDIβ* protein is the FHL1 downstream mediator that is responsible for its inhibitory role in the human lung cancer cell invasion. Collectively, these present results demonstrate that FHL1 suppression leads to *RhoGDIβ* mRNA level decrease and protein expression inhibition, and finally promotes human lung cancer cell invasion.



**Fig.4:** FHL1 ectopic expression promoted the protein and mRNA expression of *RhoGDIβ*. **A, B.** The Flag-tagged FHL1 plasmid was transfected into A549 and H1299 cell lines stably. The western blot assay was utilized to detect the expression of *RhoGDIα* and *RhoGDIβ* protein.  $\beta$ -Actin was used as a protein loading control. **C, D.** *RhoGDIβ* mRNA expression was determined by real-time polymerase chain reaction (PCR). The bars indicate the mean  $\pm$  standard deviation (SD) of 3 independent experiments. The asterisk (\*) represents a notable enhancement in comparison with vector control cells (P<0.05).



**Fig.5:** *RhoGDIβ* acted as a FHL1 downstream mediator responsible for the FHL1-inhibited human lung cancer invasion. **A.** The *RhoGDIβ* knockdown constructs were transfected into A549 (Flag-FHL1) cell lines stably. **B.** The invasion abilities of A549 (Flag-FHL1/Nonsense), A549 (Flag-FHL1/sh*RhoGDIβ*#1), and A549 (Flag-FHL1/sh*RhoGDIβ*#2) cell lines were detected (scale bar: 200 μm). **C.** The bars indicate mean  $\pm$  SD of 3 independent experiments. Student's t test was used to detect the P value. The asterisk (\*) represents a significant increase as compared to A549 (Flag-FHL1/Nonsense) transfectants (P<0.05).

## Discussion

The results of several investigations revealed that FHL1 protein expression is suppressed in a number of tumors, including breast cancer (8), gastric cancer (23), kidney cancer (24), prostate cancer (25), and liver cancer (26, 27). Niu et al. (6) found lower expression FHL1 level in the 27 lung tumors (n=30, 27/30) by using western blot. Their immunohistochemistry results showed that 100% of non-tumor lungs (80/80) expressed FHL1, while only 26.3% (21/80) of cancerous tissues stained positive for FHL1. Our results were similar to the previous studies that reported FHL1 protein is lower expressed in human lung cancers.

FHL1 exerts a tumor suppressor effect on the multiple cancers. For example, FHL1 promotes paclitaxel resistance through regulating the caspase-3 activation in the hepatic carcinoma cells (27). FHL1 overexpression gives rise to G1 and G2/M cell cycle arrest and finally decreases lung cancer cell growth (6). FHL1 influences TGF- $\beta$ -like signaling pathway activation, which leads to the inhibition of human hepatoma cell line anchorage-dependent and -independent growth *in vitro* and tumor formation in nude mice (7). All the above researches illustrated the tumor suppressor function of FHL1 in the cancer cell growth. However, other malignant functions of FHL1 is still not fully understood. In glioblastoma, FHL1 was highly expressed, and overexpression of FHL1 protein promoted the growth, migration, and invasion of glioblastoma cells *in vivo* and *in vitro* through regulating EGFR protein expression (28). In this study, the ectopic overexpression of FHL1 inhibited the invasion abilities of human lung cancer cell lines, while the migration ability was not affected.

Cell migration is the property of the live cells that is important for cell homeostasis, while cancer cell invasion means the function to migrate through the extracellular matrices and penetrate into new tissues (29). We supposed that the ectopic overexpression of FHL1 protein regulated multiple upstream factor gene expression and protein-protein interactions of cell migration. Their effects on the cell migration were finally neutralized, eventually overexpression of FHL1 showed no effect on cell migration. Moreover, we found that FHL1 overexpression promoted the mRNA and protein expression of RhoGDI $\beta$ , but not RhoGDI $\alpha$  protein expression. FHL1 overexpression might regulate the mRNA level, protein translation, or protein degradation levels of RhoGDI $\alpha$ , that ultimately had no effect on the its protein expression. In comparison with normal FHL1 overexpression human lung cancer cells, knockdown of RhoGDI $\beta$  protein reversed the invasion ability inhibition of FHL1 overexpression cells. Our results indicate that FHL1 might exert an essential role in the lung cancer progression and development.

RhoGDI $\beta$  is a member of the family of RHO guanosine diphosphate dissociation inhibitors (RhoGDIs), plays a tumor suppressor role in the diverse tumors and has been considered as an aggressive human cancer marker (15,

30). Altered RhoGDI $\beta$  expression has been observed in the multiple human cancers, including bladder cancers (18, 31), ovarian cancers (32) and lung cancer (33, 34). It has been reported that knockdown of RhoGDI $\beta$  promotes the lung cancer cell migration and invasion by regulating the PI3K/Akt pathway and MMP-9 protein expression (34). In this study, FHL1 overexpression upregulated RhoGDI $\beta$  protein expression, but had no effect on the RhoGDI $\alpha$  expression, excluding its role on the FHL1-inhibited human lung cancer invasion. Knockdown of RhoGDI $\beta$  expression completely restored the invasive ability of invasion-deficient A549 (Flag-FHL1) cells, suggesting that RhoGDI $\beta$  is a FHL1 downstream mediator responsible for its negative regulation of human lung cancer cell invasion. Due to the limitation of our study, we did not show the results of H1299 (Flag-FHL1/shRhoGDI $\beta$ ) cells to illustrate the role of RhoGDI $\beta$  for the FHL1 inhibition in human lung cancer cell invasion. In conclusion, our results showed that RhoGDI $\beta$  exerted oncogenic functions in the lung cancer cell invasion. Additionally, we also discovered that overexpression FHL1 promoted the mRNA profile of *RhoGDI $\beta$* . We suppose that *RhoGDI $\beta$*  mRNA stability or its transcription level will be regulated, and the underlying mechanism of FHL1 in regulating *RhoGDI $\beta$*  mRNA expression is still investigating in our group.

In addition, the reason underlying lower expression of FHL1 protein expression in human lung cancer is still unclear, and the molecular mechanism is worth to study in the next programme. Moreover, PI3K/Akt/mTOR pathway has been reported to be responsible for RhoGDI $\beta$  exerting oncogenic role in human lung cancer metastasis (16). Herein, we proposed a potential regulation between FHL1 and RhoGDI $\beta$  protein in the lung cancer invasion. However, it is still unknown that the downstream pathway involved in the FHL1/RhoGDI $\beta$  inhibiting lung cancer invasion.

## Conclusion

FHL1 protein was found to be downregulated in the human lung cancer patients and cell lines, which exerts a critical role in the lung cancer cell invasion. Furthermore, it was found that RhoGDI $\beta$  protein is the FHL1 protein downstream effector and is responsible for its reduction of lung cancer cell invasion. These new discoveries appear to be a potential chance to design a FHL1/RhoGDI $\beta$ -based-specific therapeutic strategy for human lung cancer treatment.

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## Authors' Contributions

X.H.; Conceived and designed the study. M.S., Y.X.;



Detected the cells' biological function, conducted the RT-PCR assays, carried out the Western blot assays, and performed the statistical analysis. X.H., M.S.; Drafted the manuscript. All authors read and approved the final version of the manuscript.

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