

Original Article

miR-25 is up-regulated in non-small cell lung cancer and promotes cell proliferation and motility by targeting FBXW7

Jie Xiang, Jun-Biao Hang, Jia-Ming Che, He-Cheng Li

Department of Thoracic Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

Received June 5, 2015; Accepted July 21, 2015; Epub August 1, 2015; Published August 15, 2015

Abstract: Increasing evidence showed that miR-25 is involved in the carcinogenesis and progression of various human cancers, while its role in non-small cell lung cancer (NSCLC) is still unclear. Here, we found that miR-25 is significantly up-regulated in NSCLC tissue samples and cell lines. Inhibition of miR-25 remarkably suppressed cell proliferation, migration and invasion in NSCLC cells, whereas enforced expression of miR-25 significantly increased NSCLC cells proliferation, migration and invasion. Moreover, we identified F-box and WD repeat domain-containing 7 (FBXW7) as a direct target of miR-25 and overexpression of FBXW7 partially attenuates the oncogenic effect of miR-25 on NSCLC cells. In conclusion, miR-25 is up-regulated in NSCLC and promotes NSCLC cells proliferation and motility partially by targeting FBXW7. Our data suggest that miR-25 might serve as a potential therapeutic target for NSCLC treatment in the future.

Keywords: miR-25, FBXW7, non-small cell lung cancer, proliferation, motility

Introduction

Lung cancer is the leading cause of cancer-related death in the world, and non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer cases [1, 2]. Despite recent progresses in diagnosis and treatment, the prognosis for NSCLC is still dismal, and the 5-year overall survival rate is about 15% [3]. Therefore, it is necessary to elucidate the underlying mechanisms of NSCLC.

MicroRNAs (miRNAs) belong to a class of highly conserved ~22-nucleotide single-stranded RNAs that epigenetically regulate protein translation through binding to the 3' untranslated region (UTR) of target mRNA [4]. miRNAs are involved in the regulation of various biological processes such as cell proliferation, apoptosis, migration and invasion [5, 6]. Accumulating evidence suggest that miRNAs can act as oncogenes or tumor suppressors in human cancer development [7] and aberrant expression of miRNAs has been observed in various human cancers such as renal cancer [8], hepatocellular carcinoma [9] and colorectal cancer [10].

Therefore, miRNAs are considered to have a great potential to be biological molecule for diagnosis, prognosis prediction and target therapy.

MiR-25 is a member of the miR-106b~25 cluster, which includes miR-106b, miR-93 and miR-25, that is located within intron 13 of the minichromosome maintenance protein7 (MCM7) gene on chromosome 7q22.1 [11, 12]. Emerging evidence has showed that up-regulation of miR-25 is associated with poor prognosis and tumor progression in several human cancers. For example, Su et al. reported that miR-25 is significantly overexpressed in hepatocellular carcinoma tissues and that patients with high miR-25 expression have a shorter overall survival than patients with low miR-25 expression [13]. Xu et al. found that the expression of miR-25 is up-regulated in esophageal squamous cell carcinoma (ESCC) tissues and over-expression of miR-25 can markedly promote migration and invasion of ESCC cells [14]. Li et al. also showed that miR-25 is overexpressed in plasma and primary tumor tissues of gastric cancer (GC) patients. In vitro assays, they demonstrated that

miR-25 inhibition significantly decreased the metastasis, invasion and proliferation of GC cells [15]. However, the expression pattern and underlying mechanism of miR-25 in NSCLC is still unclear.

In the present study, we aimed to investigate the expression level of miR-25 in NSCLC and to further explore its role in NSCLC development. We first examined the expression of miR-25 in NSCLC tissues and cell lines by using qRT-PCR. Then, we conducted *in vitro* assays to determine the biological functions of miR-25 on NSCLC cells proliferation, migration and invasion. Additionally, we identified one of the direct targets of miR-25 involved in its significance on NSCLC cells. Our research revealed miR-25 as a novel molecular associated with the development and progression of NSCLC.

Materials and methods

Tissue samples and cell lines

A total of 16 NSCLC tissue samples and paired normal tissues were collected from primary NSCLC patients who received surgery between 2012 and 2014 in our department. Tumor tissues and the paired normal tissues were frozen immediately in liquid nitrogen and stored at -80°C until use. This study was approved by the Ethics Committee of Ruijin Hospital and written informed consent was obtained from each patient. Human NSCLC cell lines A549, SPC-A-1, H460, and H661 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CCAS, China) and cultured in RPMI-1640 medium (HyClone). A normal bronchial epithelial cell line (16HBE) was purchased from the American Type Culture Collection (ATCC, USA) and cultured in DMEM medium (Gibco). The culture media were all supplemented with 10% fetal bovine serum, 50 U/ml of penicillin and 50 $\mu\text{g}/\text{ml}$ of streptomycin. All cell lines were incubated at 37°C in a humidified incubator containing 5% CO_2 .

RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from tissue samples or cell lines using Trizol reagent (Invitrogen) following the manufacturer's instruction. The quantitative real-time PCR (qRT-PCR) was performed with SYBR Green master mix (Invitrogen) using a 7500 Fast Real-Time Sequence detection system (ABI). TaqMan microRNA assays were used to evaluate the expression level of

miR-25. The primers for miR-25 were forward 5'-TCTGGTCTCCCTCACAGGAC-3' and reverse 5'-CATGGGTGCGCTACTCAC-3'. The primers for FBXW7 were forward 5'-GTCCCGAGAAGCGGTTTGATA-3' and reverse 5'-TGCTCAGGCACGT-CAGAAAAG-3'. The relative expression level of miR-25 and FBXW7 was normalized to that of the internal control U6 and GAPDH, respectively, by using the $2^{-\Delta\Delta\text{Ct}}$ method. All experiments were conducted in triplicate.

Plasmid construction and cell transfection

miR-25 mimics/inhibitor (miR-25/miR-25-I) and its non-specific control miRNA (miR-NC) were purchased from RiboBio (Guangzhou). The FBXW7 cDNA was cloned into pcDNA3.1 to construct the FBXW7 expression plasmid. For luciferase reporter assay, the 3'-UTR of FBXW7 which contains putative binding sites of miR-25 was amplified by PCR and subcloned into psiCHECK-2 vector within XhoI and NotI restriction sites (Promega). Mutation in the miR-25-binding site of FBXW7 was introduced by whole-plasmid amplification in the seed region of miR-25 (NEB). Cell transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

Luciferase reporter assay

For luciferase reporter assay, A549 cells were grown in 24-well plates and co-transfected with miR-25 or control (miR-NC) mimics and wild-type (WT) or mutated (Mut) 3'-UTR of FBXW7. After transfected for 48 h, cells were collected and the relative luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). The experiment was independently repeated three times.

Cell proliferation

Cell proliferation of A549 cells were measured by using the Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacturer's protocol. Briefly, after effective transfection, A549 cells were plated into 96-well plates at a density of 5×10^3 cells per well and incubated in RPMI-1640 medium supplemented with 10% FBS for 24, 48, 72 and 96 h, respectively. After that, 10 μl CCK-8 liquid was added to the test well and incubated for 3 h. Absorbance was then measured at a wavelength of 450 nm. Three independent experiments were performed.

miR-25 expression in NSCLC

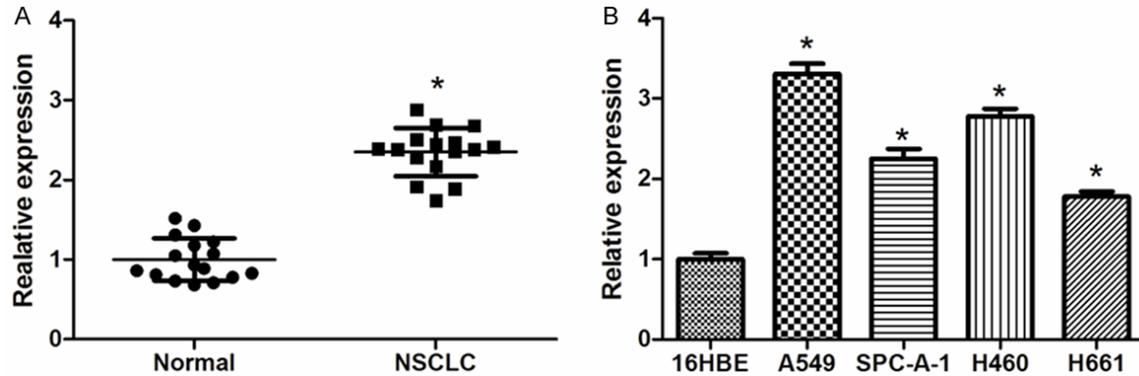


Figure 1. miR-25 is up-regulated in NSCLC tissues and cell lines. A. Relative expression level of miR-25 in NSCLC tissue samples and adjacent normal tissue samples detected by qRT-PCR. B. Relative expression level of miR-25 in NSCLC cell lines and normal bronchial epithelial cell line 16HBE detected by qRT-PCR. Data are expressed as mean \pm SD from three independent experiments. * $P < 0.05$.

Cell migration and invasion assays

Cell migration and invasion assays were determined using transwell insert chambers. Briefly, the transfected cells were collected and seeded into the upper chamber of the Transwell insert (with Matrigel for invasion and without for migration). RPMI-1640 with 10% FBS was added to the lower chamber as the chemoattractant. After incubation at 37°C for 24 h, cells in the upper chambers were removed carefully with a cotton swab. Cells which migrated or invaded through the membrane were fixed with 4% polyoxymethylene, stained with 0.2% crystal violet and counted under a light microscope. Experiments were repeated three times independently.

Western blot

Total proteins were extracted from treated cells with RIPA buffer (Beyotime) supplemented with protease inhibitors (Roche). Protein samples were separated by 10% SDS-PAGE and then electrotransferred to PVDF membranes (Bio-Rad). After blocking with 5% non-fat milk at room temperature for 1 h, the membranes were incubated with primary antibodies (Abcam) at 4°C overnight, followed by secondary antibodies at 37°C for 1 h. protein bands were visualized using ECL system (Pierce). GAPDH was used as the internal control. Each experiment was repeated in triplicate.

Statistical analysis

Data were expressed as mean \pm SD from three independently experiments and analyzed by using SPSS version 17.0 software. The significance of differences between groups was com-

pared by Student's t test (two groups) or one-way analysis of variance (ANOVA, more than two groups). $P < 0.05$ was considered statistically significant.

Results

miR-25 is up-regulated in NSCLC tissues and cell lines

The expression level of miR-25 in NSCLC tissue samples and corresponding normal tissue samples was analyzed by using qRT-PCR. Data showed that miR-25 is up-regulated in NSCLC tissues in comparison with that in corresponding normal tissues (Figure 1A). Furthermore, compared with normal bronchial epithelial cell line 16HBE, the expression of miR-25 was also significantly up-regulated in NSCLC cell lines (Figure 1B).

Inhibition of miR-25 suppresses NSCLC cells proliferation and motility

To investigate the effect of miR-25 on NSCLC cells proliferation and motility, A549 cells were transfected with miR-25 inhibitor (miR-25-I) or miR-NC. After effective transfection, miR-25 expression was confirmed by qRT-PCR (Figure 2A). Results of CCK-8 assay showed that inhibition of miR-25 significantly suppressed NSCLC cells proliferation (Figure 2B). Then, cell motility was measured by transwell migration and invasion assays, and inhibition of miR-25 effectively suppressed NSCLC cells migration and invasion (Figure 2C, 2D). These results reveal that inhibition of miR-25 suppresses NSCLC cells proliferation and motility.

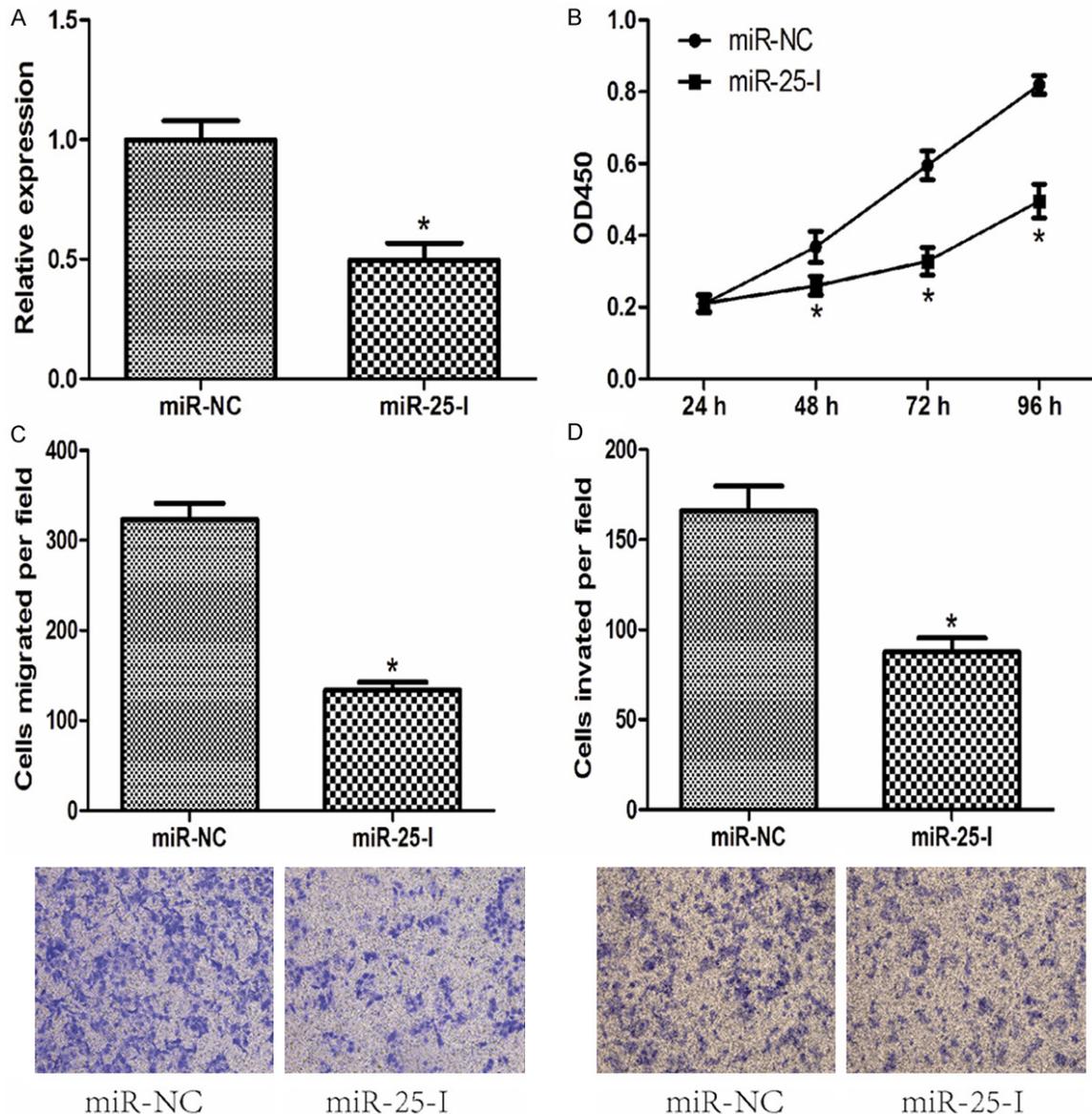


Figure 2. Inhibition of miR-25 suppresses NSCLC cells proliferation and motility. A. A549 cells were transfected with miR-25 inhibitor (miR-25-I) or miR-NC, and the relative expression level of miR-25 was detected by qRT-PCR. B. Cell proliferation of pretreated A549 cells examined by CCK-8 assay. C, D. Cell motility of pretreated A549 cells determined by transwell migration and invasion assays. Data are expressed as mean \pm SD from three independent experiments. * $P < 0.05$.

FBXW7 is a direct target of miR-25

TargetScan 6.2 was used to explore the potential target of miR-25 in NSCLC. FBXW7, a tumor suppressor in human tumorigenesis, was predicted as a target of miR-25 (Figure 3A). To confirm that, wild type (WT) or mutated (Mut) FBXW7 3'UTR was cloned into luciferase reporter vectors and luciferase activity assay was conducted. We found that overexpression of miR-25 significantly suppressed WT but not Mut 3'UTR of FBXW7 (Figure 3B). In addition,

our results of western blot showed that inhibition of miR-25 significantly increased the protein level of FBXW7, while overexpression of miR-25 significantly reduced the protein level of FBXW7 (Figure 3C, 3D). These data suggest that FBXW7 is a direct target of miR-25.

FBXW7 overexpression partially attenuates the oncogenic effect of miR-25

To further investigate whether overexpression of FBXW7 could attenuate the oncogenic effect

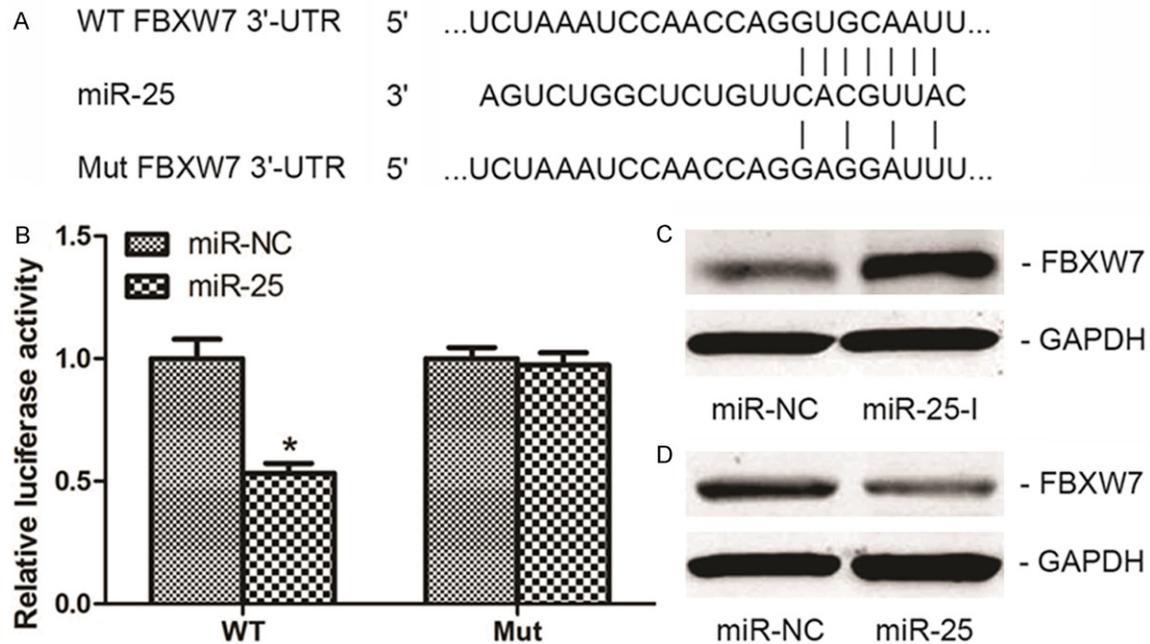


Figure 3. FBXW7 is a direct target of miR-25. **A.** The potential miR-25 targeting site in FBXW7 3'-UTR and the mutated sequences. **B.** The relative luciferase activity was measured in A549 cells after co-transfected with miR-25 or miR-NC with wild-type (WT) or mutated (Mut) 3'-UTR of FBXW7. **C.** The protein level of FBXW7 in A549 cells after transfected with miR-25 inhibitor (miR-25-I) or miR-NC. **D.** The protein level of FBXW7 in A549 cells after transfected with miR-25 or miR-NC. Data are expressed as mean \pm SD from three independent experiments. * $P < 0.05$.

ct of miR-25 on NSCLC cells, the pcDNA3.1-FBXW7 plasmid or vector was transfected into A549 cells and the effect was validated by qRT-PCR (**Figure 4A**). Results of CCK8 assays (**Figure 4B**), transwell migration and invasion assays (**Figure 4C, 4D**) all showed that supplement of FBXW7 could significantly attenuate the oncogenic effect of miR-25 on NSCLC cells. These data suggest that miR-25 promotes NSCLC cells proliferation and motility partially by targeting FBXW7.

Discussion

Lung cancer is one of the most life-threatening tumors, with approximately two million new cases diagnosed worldwide annually [1]. Despite the improvements in therapeutic modalities, the 5-year survival rate of NSCLC patients is still around 15% [3]. In recent years, a large number of miRNAs have been detected to be aberrantly expressed in NSCLC tissues and suggest that miRNAs may play essential roles in NSCLC development. For example, microRNA-27b is reported to be significantly downregulated in NSCLC tissues and can inhibit cell growth and invasion by downregulating Sp1 in NSCLC cells [16]. miR-195 is also demonstrated to inhibit cell proliferation, migration, and

invasion of NSCLC cells by targeting MYB [17]. Additionally, microRNA-141 promotes proliferation of NSCLC cells by regulating expression of PHLPP1 and PHLPP2 [18].

In this study, we explored the expression level of miR-25 and its underlying mechanism in NSCLC for the first time. By using qRT-PCR, we demonstrated that miR-25 is up-regulated in NSCLC tissues and cell lines when comparison with corresponding normal tissues and normal bronchial epithelial cell line 16HBE, respectively. We also found that inhibition of miR-25 significantly suppressed NSCLC cells proliferation, migration and invasion. On the contrary, enforced expression of miR-25 markedly increased NSCLC cells proliferation, migration and invasion. These results suggest that miR-25 is involved in the development and progression of NSCLC.

In addition, we found that FBXW7 is a potential target of miR-25 in NSCLC predicted by TargetScan 6.2 and we verified that by luciferase reporter assay. FBXW7 (F-box and WD repeat domain-containing 7) also known as FBW7, a well-studied F-box-containing protein in the SCF (SKP1-CUL1-F-box protein) E3 ligase complex, determines target specificity through

miR-25 expression in NSCLC

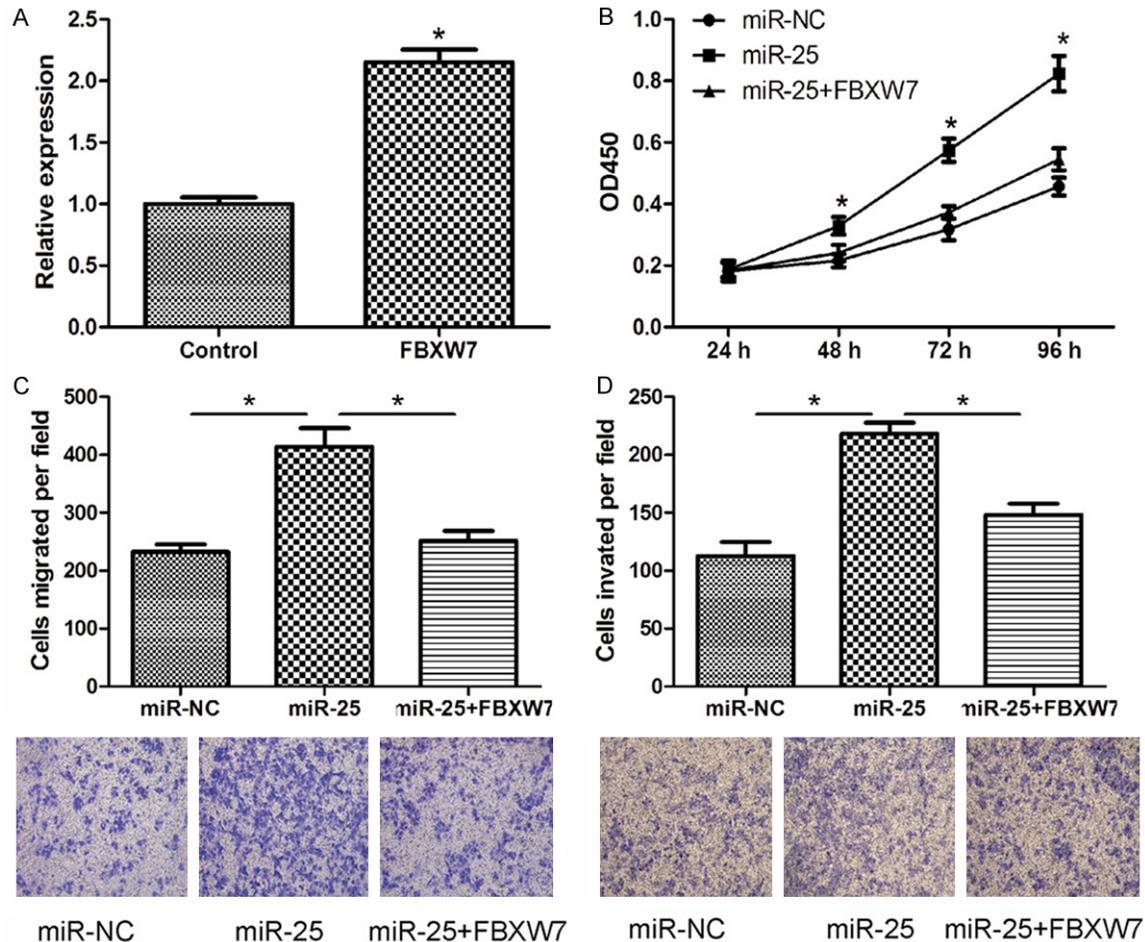


Figure 4. FBXW7 overexpression partially attenuates the oncogenic effect of miR-25. A. A549 cells were transfected with pcDNA3.1-FBXW7 plasmid or vector, and the relative mRNA level of FBXW7 was detected by qRT-PCR. B. A549 cells were co-transfected with miR-25 and pcDNA3.1-FBXW7 plasmid or vector, and cell proliferation was examined by CCK-8 assay. C, D. Cell motility of pretreated A549 cells determined by transwell migration and invasion assays. Data are expressed as mean \pm SD from three independent experiments. * $P < 0.05$.

recognition and binding of target proteins for ubiquitination and degradation [19]. Several lines of evidence suggest that FBXW7 is a putative tumor suppressor in human tumorigenesis. Li et al demonstrated that FBXW7 overexpression inhibited cell proliferation and promoted apoptosis in osteosarcoma cells, and that patients with high expression of FBXW7 had a better prognosis [20]. Ibusuki et al. showed that in breast cancer, patients with lower FBXW7 expression had a poorer prognosis and silencing FBXW7 upregulated cell proliferation and G1-S transition [21]. Besides, Naganawa et al. reported that decreased expression of FBXW7 is correlated with poor prognosis in patients with esophageal squamous cell carcinoma [22]. In our present study, we confirmed that FBXW7 is a direct target of miR-25 in NSCLC and demonstrated that FBXW7 overex-

pression partially attenuates the oncogenic effect of miR-25 on NSCLC cell line A549.

In conclusion, we found that miR-25 is significantly up-regulated in NSCLC tissues and cell lines. By partially inhibiting the expression of FBXW7, overexpression of miR-25 promotes cell proliferation and motility in NSCLC cells. These results suggest that miR-25 might represent a potential therapeutic target for NSCLC treatment in the future.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. He-Cheng Li, Department of Thoracic Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China. E-mail: hcl1968@sina.com

References

- [1] Siegel R, Naishadham D and Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013; 63: 11-30.
- [2] Ettinger DS, Akerley W, Bepler G, Blum MG, Chang A, Cheney RT, Chirieac LR, D'Amico TA, Demmy TL, Ganti AK, Govindan R, Grannis FW Jr, Jahan T, Jahanzeb M, Johnson DH, Kessinger A, Komaki R, Kong FM, Kris MG, Krug LM, Le QT, Lennes IT, Martins R, O'Malley J, Osarogiagbon RU, Otterson GA, Patel JD, Pisters KM, Reckamp K, Riely GJ, Rohren E, Simon GR, Swanson SJ, Wood DE and Yang SC. Non-small cell lung cancer. *J Natl Compr Canc Netw* 2010; 8: 740-801.
- [3] Henschke CI, Yankelevitz DF, Libby DM, Pasmantier MW, Smith JP and Miettinen OS. Survival of patients with stage I lung cancer detected on CT screening. *N Engl J Med* 2006; 355: 1763-1771.
- [4] Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 2005; 6: 376-385.
- [5] Li X, Li H, Zhang R and Liu J. MicroRNA-449a inhibits proliferation and induces apoptosis by directly repressing E2F3 in gastric cancer. *Cell Physiol Biochem* 2015; 35: 2033-2042.
- [6] Yang C, Ning S, Li Z, Qin X and Xu W. miR-22 is down-regulated in esophageal squamous cell carcinoma and inhibits cell migration and invasion. *Cancer Cell Int* 2014; 14: 138.
- [7] Zhang B, Pan X, Cobb GP and Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol* 2007; 302: 1-12.
- [8] Yang FQ, Zhang HM, Chen SJ, Yan Y and Zheng JH. MiR-506 Is Down-Regulated in Clear Cell Renal Cell Carcinoma and Inhibits Cell Growth and Metastasis via Targeting FLOT1. *PLoS One* 2015; 10: e0120258.
- [9] Sui C, Xu F, Shen W, Geng L, Xie F, Dai B, Lu J, Zhang M and Yang J. Overexpression of miR-218 inhibits hepatocellular carcinoma cell growth through RET. *Tumour Biol* 2015; 36: 1511-1518.
- [10] Zhang J, Luo X, Li H, Yue X, Deng L, Cui Y and Lu Y. MicroRNA-223 functions as an oncogene in human colorectal cancer cells. *Oncol Rep* 2014; 32: 115-120.
- [11] Petrocca F, Vecchione A and Croce CM. Emerging role of miR-106b-25 miR-17-92 clusters in the control of transforming growth factor beta signaling. *Cancer Res* 2008; 68: 8191-8194.
- [12] Savita U and Karunagaran D. MicroRNA-106b-25 cluster targets beta-TRCP2, increases the expression of Snail and enhances cell migration and invasion in H1299 (non small cell lung cancer) cells. *Biochem Biophys Res Commun* 2013; 434: 841-847.
- [13] Su ZX, Zhao J, Rong ZH, Geng WM, Wu YG and Qin CK. Upregulation of microRNA-25 associates with prognosis in hepatocellular carcinoma. *Diagn Pathol* 2014; 9: 47.
- [14] Xu X, Chen Z, Zhao X, Wang J, Ding D, Wang Z, Tan F, Tan X, Zhou F, Sun J, Sun N, Gao Y, Shao K, Li N, Qiu B and He J. MicroRNA-25 promotes cell migration and invasion in esophageal squamous cell carcinoma. *Biochem Biophys Res Commun* 2012; 421: 640-645.
- [15] Li BS, Zuo QF, Zhao YL, Xiao B, Zhuang Y, Mao XH, Wu C, Yang SM, Zeng H, Zou QM and Guo G. MicroRNA-25 promotes gastric cancer migration, invasion and proliferation by directly targeting transducer of ERBB2, 1 and correlates with poor survival. *Oncogene* 2015; 34: 2556-2565.
- [16] Jiang J, Lv X, Fan L, Huang G, Zhan Y, Wang M and Lu H. MicroRNA-27b suppresses growth and invasion of NSCLC cells by targeting Sp1. *Tumour Biol* 2014; 35: 10019-10023.
- [17] Yongchun Z, Linwei T, Xicai W, Lianhua Y, Guangqiang Z, Ming Y, Guanjian L, Yujie L and Yunchao H. MicroRNA-195 inhibits non-small cell lung cancer cell proliferation, migration and invasion by targeting MYB. *Cancer Lett* 2014; 347: 65-74.
- [18] Mei Z, He Y, Feng J, Shi J, Du Y, Qian L, Huang Q and Jie Z. MicroRNA-141 promotes the proliferation of non-small cell lung cancer cells by regulating expression of PHLPP1 and PHLPP2. *FEBS Lett* 2014; 588: 3055-3061.
- [19] Wang Z, Inuzuka H, Zhong J, Wan L, Fukushima H, Sarkar FH and Wei W. Tumor suppressor functions of FBW7 in cancer development and progression. *FEBS Lett* 2012; 586: 1409-1418.
- [20] Li Z, Xiao J, Hu K, Wang G, Li M, Zhang J and Cheng G. FBXW7 acts as an independent prognostic marker and inhibits tumor growth in human osteosarcoma. *Int J Mol Sci* 2015; 16: 2294-2306.
- [21] Ibusuki M, Yamamoto Y, Shinriki S, Ando Y and Iwase H. Reduced expression of ubiquitin ligase FBXW7 mRNA is associated with poor prognosis in breast cancer patients. *Cancer Sci* 2011; 102: 439-445.
- [22] Naganawa Y, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Katada T, Tanaka T, Shiozaki M, Fujii Y and Takeyama H. Decreased expression of FBXW7 is correlated with poor prognosis in patients with esophageal squamous cell carcinoma. *Exp Ther Med* 2010; 1: 841-846.