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Original Article

MiR-25 reduces PC-9/BB4 cell apoptosis sensitivity induced by gefitinib through downregulating BIM

Yi Tang, Gaoming Xiao, Yuejun Chen, Yu Deng

The 1st Department of Thoracic Surgery, The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, P. R. China

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Abstract: Bcl-2 interacting mediator of cell death (BIM) that mediated apoptosis was found to be upregulated in drug resistant lung cancer cell line. It was showed that miR-25 significantly elevated in lung cancer tissue from non-small cell lung cancer patients. This study investigated the relationship between lung cancer drug resistance and abnormal expression of miR-25 and BIM. Drug sensitive PC-9 cells and resistant PC-9/BB4 cells were cultured *in vitro* and treated with gefitinib at 0.015 μM. Cell apoptosis was detected by flow cytometry. MiR-25 and BIM expressions were compared. The targeted relationship between miR-25 and BIM was confirmed by dual luciferase reporter assay. PC-9/BB4 cells treated by gefitinib at 0.015 μM were divided into six groups, including control, inhibitor NC, miR-25 inhibitor, scramble-pMD18-T, BIM-pMD18-T, and miR-25 + BIM-pMD18-T. Cell viability was determined by CCK8 assay. Caspase activity was tested using spectrophotometry. MiR-25 expression in PC-9/BB4 cells was significantly higher, while BIM level was obviously lower than that in PC-9 cells. Gefitinib markedly induced PC-9 cell apoptosis and upregulated BIM expression, whereas showed poor impact on PC-9/BB4 cell apoptosis and BIM level. MiR-25 suppressed BIM expression through targeting its 3'-UTR. MiR-25 inhibitor and/or BIM overexpression significantly upregulated BIM expression, enhanced caspase-9 and caspase-3 activities, declined cell viability, and increased cell apoptosis. MiR-25 elevated, while BIM reduced in PC-9/BB4 cells. MiR-25 suppressed PC-9/BB4 cell apoptosis induced by gefitinib through targeting BIM, which may play a role in PC-9/BB4 cell drug resistance.

Keywords: miR-25, BIM, lung cancer, gefitinib, apoptosis, drug resistance

Introduction

Lung cancer is a common malignant tumor in clinic as the leading cause of cancer death, which is a severe threat to human life and health [1]. Non-small cell lung cancer (NSCLC) is the most common pathological type of lung cancer, accounting for about 70% to 80% of all lung cancer [2]. At present, surgical resection is still the preferred treatment for NSCLC in early stage. Because of conceals onset, unconspicuous early symptom, and no sensitive specific indicators, most patients have been in advanced stage losing the best opportunity for surgery. These patients mainly receive comprehensive treatment including radiotherapy, chemotherapy, and surgery. Even so, the comprehensive treatment fails to significantly improve the prognosis of patients [3]. Epidermal growth factor receptor (EGFR) upregulates in most tumors originated from epithelium, and its excessive expression is related to tumor cell proliferation, apoptosis, and invasion [4, 5]. Recently, it was proved that molecular therapy targeted EGFR played an important role in lung cancer treatment, of which EGFR tyrosine kinase inhibitor (TKI) has been approved by many countries as first-line therapy for lung cancer in advanced stage [6]. Numerous studies found that almost all NSCLC patients may appear recurrence or progress after treated by gefitinib for 6 to 12 months, suggesting acquired drug resistance [7].

BIM, also known as BCL2L11 (Bcl-2-like protein 11), belongs to the subfamily of Bcl-2 protein family contains BH3-only domain structure and plays a key role in triggering cell apoptosis [8, 9]. It was found that BIM protein downregulation was associated with NSCLC pathogenesis [10]. Various studies demonstrated that chemotherapy drug induced lung cancer cell apop-

tosis obviously by increasing BIM level, indicating the function of BIM in mediating cell apoptosis induced by chemotherapy drugs [11-13]. Other studies showed that BIM level was obviously higher in drug resistant lung cancer cell line compared with drug sensitive cancer cell line under the induction of gefitinib, revealing that BIM may be involved in acquired drug resistance of lung cancer cells [14]. It has been proved that miR-25 level markedly upregulated in NSCLC tumor tissue, indicating that miR-25 may promote canceration of NSCLC [15, 16]. Bioinformatics analysis demonstrated the good complimentary relationship between miR-25 and the 3'-UTR of BIM. This study explored the relationship between lung cancer drug resistance and abnormal expression of miR-25 and BIM.

Materials and methods

Main reagents and materials

Drug sensitive NSCLC cell line PC-9 and drug resistant cell line PC-9/BB4 were provided by the Cancer Institute of Shanghai Tongji University. DMEM, FBS, and penicillin-streptomycin were obtained from Gibco. Trizol and lipofectamine 2000 were purchased from Invitrogen. Reverse transcription kit ReverTra Ace qPCR RT kit was gotten from Toyobo. SYBR Green Real-Time PCR Master Mixes were form Life Technologies. MiR-25 nucleotide and PCR primers were synthetized by Ribobio. Mouse anti human BIM primary antibody was from Santa Cruz. Rabbit anti human Cleaved Caspase-3 and Cleaved Caspase-9 were purchased from Cell Signaling. BCA protein quantification kit was from Boster. RIPA lysis, Annexin V/PI apoptosis detection kit, and Caspase enzyme activity detection kit were from Beyotime. Dual-luciferase® Reporter Assay System and pGL3-promoter plasmid were from Promega. Gefitinib was bought from LC laboratories. GloMax 20/20 light detector was from Promega. Flow cytometry instrument FC 500 MCL was bought from Beckman. ABI 7500 real-time PCR was from Life technologies. Electrophoresis apparatus was bought from Bio-Rad. Multifunctional microplate reader was from BioTek.

Cell culture

NSCLC cell line PC-9 and PC-9/BB4 were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and maintained at 37°C and 5% CO_2. The medium was changed every two days. The cells were used for experiments when the fusion reached 70% to 80%.

CCK-8 assay

The cells were seeded in 96-well plate at 1×10^4/well for 24 h. Then the cells were treated with gefitinib at 0, 0.01, 0.1, 1.0, and 10.0 μM for 48 h. Next, the medium was changed to 100 μl medium containing 10 μl CCK-8 reagent at 37°C for 4 h. At last, the plate was measured on microplate reader at 450 nm. Each group was repeated for six replicates. Relative viability = $(OD_{test}-OD_{blank})/OD_{control}\times100\%$.

Luciferase reporter gene vector construction

The 3'-UTR of BIM gene were amplified based on HEK293 cell genome. PCR product was recycled and double digested by XbaI/NotI. Then it was connected to luciferase reporter vector pGL-3M to transform DH5α competent cells. After colony PCR, the positive clone was screened and the plasmid with correct sequence was applied for cell transfection.

Luciferase reporter gene assay

The HEK293 cells were transfected with 200 ng pGL3-BIM-3'UTR, 50 nmol microRNA nucleotide fragment, and 50 ng pRL-TK mixture mediated by Lipofectamine 2000. After incubated for 4-6 h, Opti-MEM medium was changed to DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin for another 48 h. Luciferase activity was detected according to the manual provided by the kit. After washed by PBS for twice, the cells were added with 100 μl PLB for 15 min and centrifuged at 10,000 g for 5 min. The supernatant was added with 50 μl LAR II and tested immediately in chemiluminescence apparatus for fluorescence I. Then the solution was added with 50 μl Stop&Glo solution to test fluorescence II. The ratio of fluorescence I and fluorescence II was treated as relative expression level of luciferase activity.

Overexpression vector construction and cell transfection

pMD18-T was selected as plasmid vector, while EcoIR1 and Sal1 were chosen as restriction

endonuclease to amplify BIM gene. The primers for BIM were designed according to the cDNA sequence of BIM in Gene Bank. Forward, 5'-GACGAATTCATGGCAAAGCAACCT-3', reverse, 5'-GACGTCGACTTAATCAGGTGGAAG-3'. Gel electrophoresis was performed to determine the target fragment at 519 bp. The target fragment was recycled and transform into competent cell BJ5183 after connecting to vector. Positive clones were screened upon ampicillin resistant solid board and amplified to extract plasmid containing target fragment. The inserted plasmid was sequenced to confirm the correctness. Negative control plasmid (Scramble-pMD18-T vector) or BIM overexpression plasmid (BIM-pMD18-T vector) was transfected into PC-9/BB4 cells using calcium phosphate transfection method. Stably transfected positive cell clones were screened by G418. Transfected PC-9/BB4 cells were treated by gefitinib at 0.015 μM and divided into six groups, including control, inhibitor NC, miR-25 inhibitor, scramble-pMD18-T, BIM-pMD18-T, and miR-25 + BIM-pMD18-T. The cells were collected after 48 h for the following experiments.

qRT-PCR

Total RNA was extracted from cells using Trizol. The cells were treated by 1 ml Trizol and extracted by 200 μl chloroform. After 1 ml isopropanol sediment and 1 ml 70% ethanol washing, total RNA was diluted in DEPC water. Then the RNA was reversely transcripted to cDNA by ReverTra Ace qPCR RT Kit at 37°C for 15 min and 98°C for 5 min. The reverse transcription system contained 2 μg total RNA, 4 μL RT Buffer (5×), 1 μL oligo dT + Random primer Mix, 1 μL RT Enzyme Mix, 1 μL RNase inhibitor, and ddH_2O. The PCR primers used were as follows. $miR\text{-}25P_F$: 5'-CGGCGGCATTGCACTTGGTCTC-3', $miR\text{-}25P_R$: 5'-GTGCAGGGTCCGAGGT-3'; $U6P_F$: 5'-ATTGGAACGATACAGAGAAGATT-3', $U6P_R$: 5'-GGAACGCTTCACGAATTTG-3'; $BIMP_F$: 5'-TAAGTTCTGAGTGTGACCGAGA-3', $BIMP_R$: 5'-GCTCTGTCTGTAGGGAGGTAGG-3'; $\beta\text{-actinP}_F$: 5'-GAACCCTAAGGCCAAC-3', $\beta\text{-actinP}_R$: 5'-TGTCACGCACGATTTCC-3'. The PCR reaction system (10 μl of total volume) was composed of 5 μl 2×SYBR Green Mixture, 1 μl forward and reverse primer at 2.5 μM, 1 μl cDNA, and 3 μl ddH_2O. The reaction was performed on ABI 7500 for 40 cycles, including 95°C for 15 s, 60°C for 30 s, and 74°C for 30 s. Each test was repeated for three times.

Western blot

Total protein was extracted from the cells by RIPA on ice and centrifuged at 10000 g for 30 min. After quantified by BCA method, a total of 50 μg protein was separated by 8% SDS-PAGE for 3 h and transferred to PVDF membrane at 200 mA for 100 min. After blocked by 5% skim milk at room temperature for 60 min, the membrane was incubated in primary antibody at 4°C overnight. After washed by PBST, the membrane was further incubated in HRP labeled secondary antibody at room temperature for 60 min. At last, the membrane was treated by ECL chemiluminiscence reagent and developed. Quantity One was applied to analyze the protein bind.

Caspase-3 and Caspase-9 activities detection

The Peptide nucleic acid (pNA) (10 mM) was diluted to 0, 10, 20, 50, 100, and 200 μM with standard dilution solution, respectively. Their absorbance at 405 nm was tested to draw the standard curve. The cells were digested and centrifuged at 600 g for 5 min. After washed by PBS, a total of 2×10^6 cells were added to 100 μl lysis at 4°C for 15 min. After centrifuged at 18,000 g for 10 min, the supernatant was moved to a precooled Ep tube. For Caspase-3 enzyme activity detection, a total of 10 μl Ac-DEVD-pNA (2 mM) was added to the solution and incubated at 37°C for 2 h to test absorbance at 405 nm. For Caspase-9 enzyme activity detection, a total of 10 μl Ac-LEHD-pNA (2 mM) was added to the solution and incubated at 37°C for 2 h to test the absorbance at 405 nm.

Flow cytometry detection of cell apoptosis

The cells were digested by enzyme and collected. After resuspended in 195 μL binding buffer, the cells were added with 5 μl Annexin V-FITC and 10 μl PI at room temperature avoid of light for 15 min. Then the cells were tested on flow cytometry immediately after incubated on ice for 5 min.

Statistical analysis

SPSS 18.0 software was applied for data analysis. Measurement data was presented as mean ± standard deviation and compared by t test. $P < 0.05$ was depicted as significant difference.

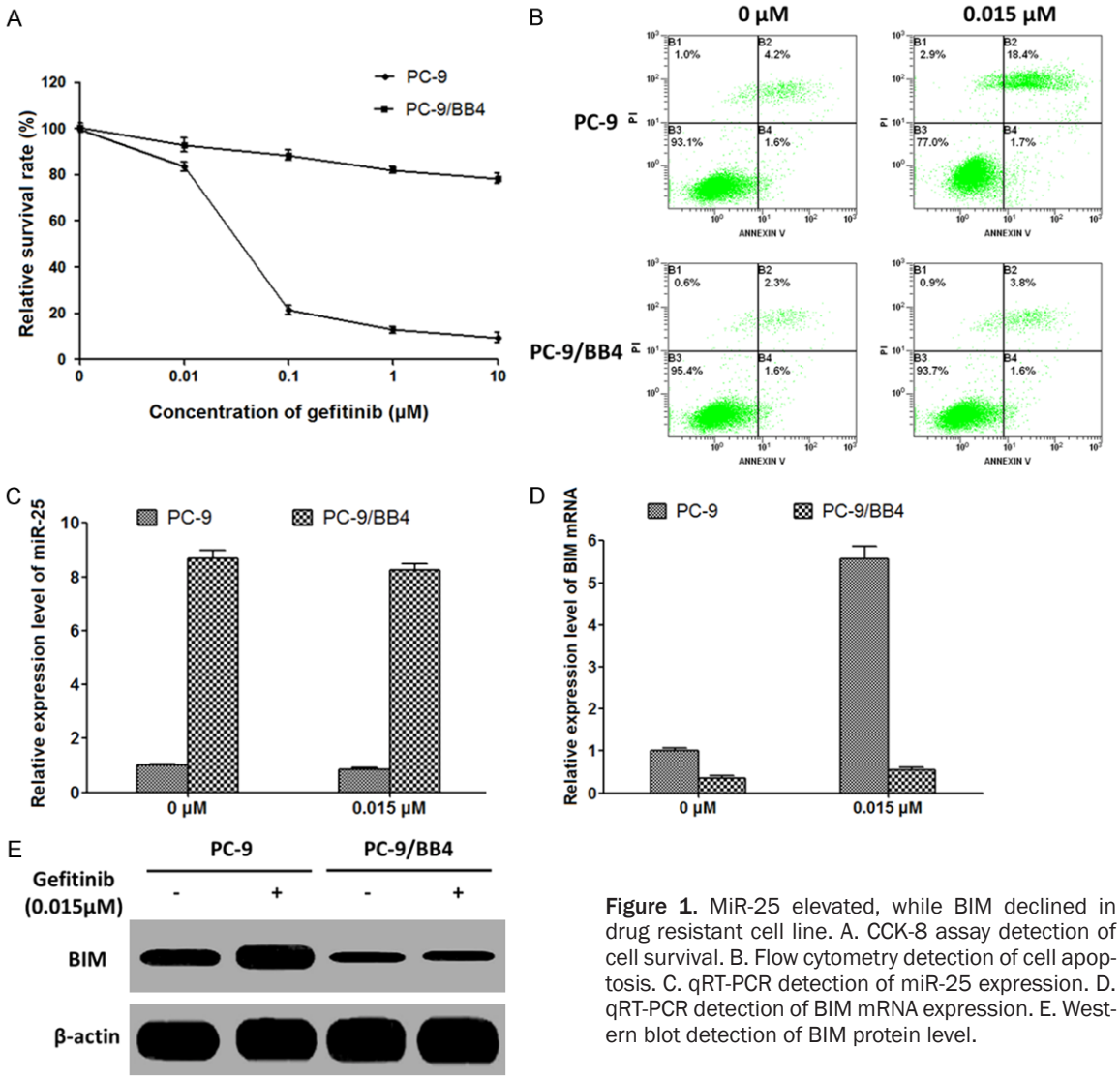

Figure 1. MiR-25 elevated, while BIM declined in drug resistant cell line. A. CCK-8 assay detection of cell survival. B. Flow cytometry detection of cell apoptosis. C. qRT-PCR detection of miR-25 expression. D. qRT-PCR detection of BIM mRNA expression. E. Western blot detection of BIM protein level.

Results

MiR-25 elevated, while BIM declined in drug resistant cell line

After treated with different concentrations of gefitinib, PC-9 cell viability significantly declined with IC50 at 0.043 μM. Drug resistant PC-9/BB4 cell line was cultured based on PC-9 cells treated by gefitinib for long-term, as it showed lower sensitivity to gefitinib compared with PC-9 cells (**Figure 1**). The relative viability of PC-9 cells was 80% when treated by gefitinib at 0.015 μM. Thus, this study used 0.015 μM as the gefitinib concentration in the following experiment. Compared with untreated group, 0.015 μM gefitinib obviously induced PC-9 cell apoptosis but failed to exhibit significant impact on PC-9/BB4 cells (**Figure 1B**). BIM expression markedly increased in PC-9 cells after treated by gefitinib, while was insensitive in PC-9/BB4 cells (**Figure 1D** and **1E**). Further analysis demonstrated that miR-25 level in PC-9 cells was significantly lower than that in PC-9/BB4 cells, whereas gefitinib intervention showed no apparent impact on miR-25 expression (**Figure 1C**). It suggested that BIM downregulation in PC-9/BB4 cells might be affected by miR-25 abnormal elevation, that is miR-25 upregulation and BIM declination may play a role in mediating cell drug resistance.

MiR-25 regulated BIM expression

Based on the prediction by microRNA.org, miR-25 showed good complimentary targeted rela-

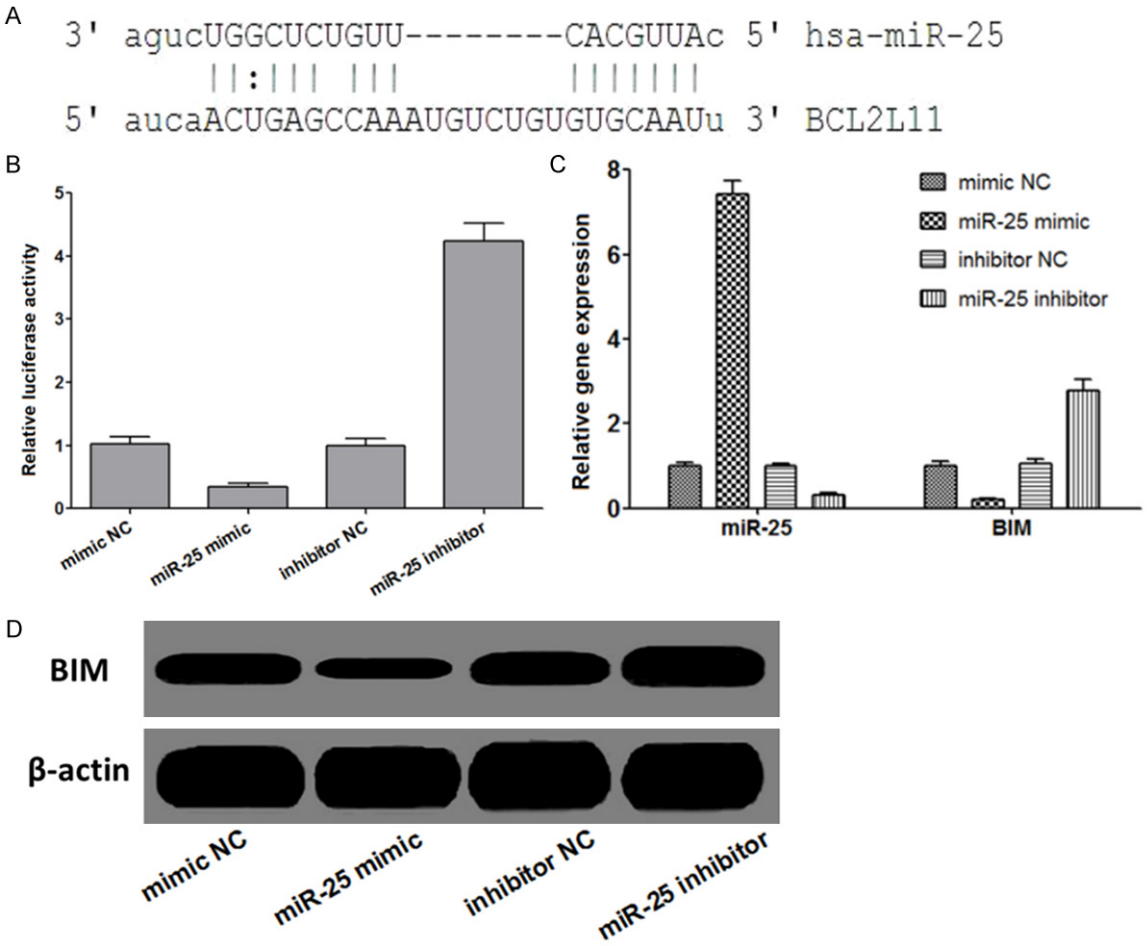

Figure 2. MiR-25 targeted regulated BIM expression. A. miR-25 targeted the 3'-UTR of BIM. B. Dual-luciferase reporter assay. C. qRT-PCR detection of miR-25 and BIM gene expression. D. Western blot detection of BIM protein level.

tionship with the 3'-UTR of BIM (**Figure 2A**). Luciferase assay revealed that miR-25 elevation apparently decreased the relative luciferase activity in HEK293 cell lysis, whereas miR-25 reduction obviously enhanced its relative luciferase activity (**Figure 2B**). It indicated that miR-25 can target on the 3'-UTR of BIM mRNA and regulate its expression. Further investigation demonstrated that miR-25 upregulation or declination significantly reduced and increased BIM level in PC-9/BB4 cells, respectively, confirming that BIM was targeted regulated by miR-25 (**Figure 2C** and **2D**).

MiR-25 targeted BIM weakened PC-9/BB4 cell apoptosis sensitivity

We further discussed the impact of BIM and miR-25 abnormal expression on PC-9/BB4 cell drug resistance. MiR-25 inhibitor and/or BIM overexpression transfection significantly upregulated BIM expression (**Figure 3A** and **3B**), enhanced caspase-9 and caspase-3 activities (**Figure 3B** and **3C**), declined cell viability (**Figure 3D**), and increased cell apoptosis (**Figure 3E**).

Discussion

Lung cancer is the largest malignant fastest threat to human health and life featured as the fastest growing morbidity and mortality. It has become the leading cause of death from cancer worldwide [1]. Following industry development and atmospheric environment deterioration in the past twenty years, the morbidity and mortality of lung cancer in our country kept on increasing. It was reported that the lung cancer cases in our country grew from 600,000 to 700,000, the morbidity grew from 46.1/100,000 to 50.5/100,000, while case fatality rate grew from 37.0/100,000 to 42.3/100,000

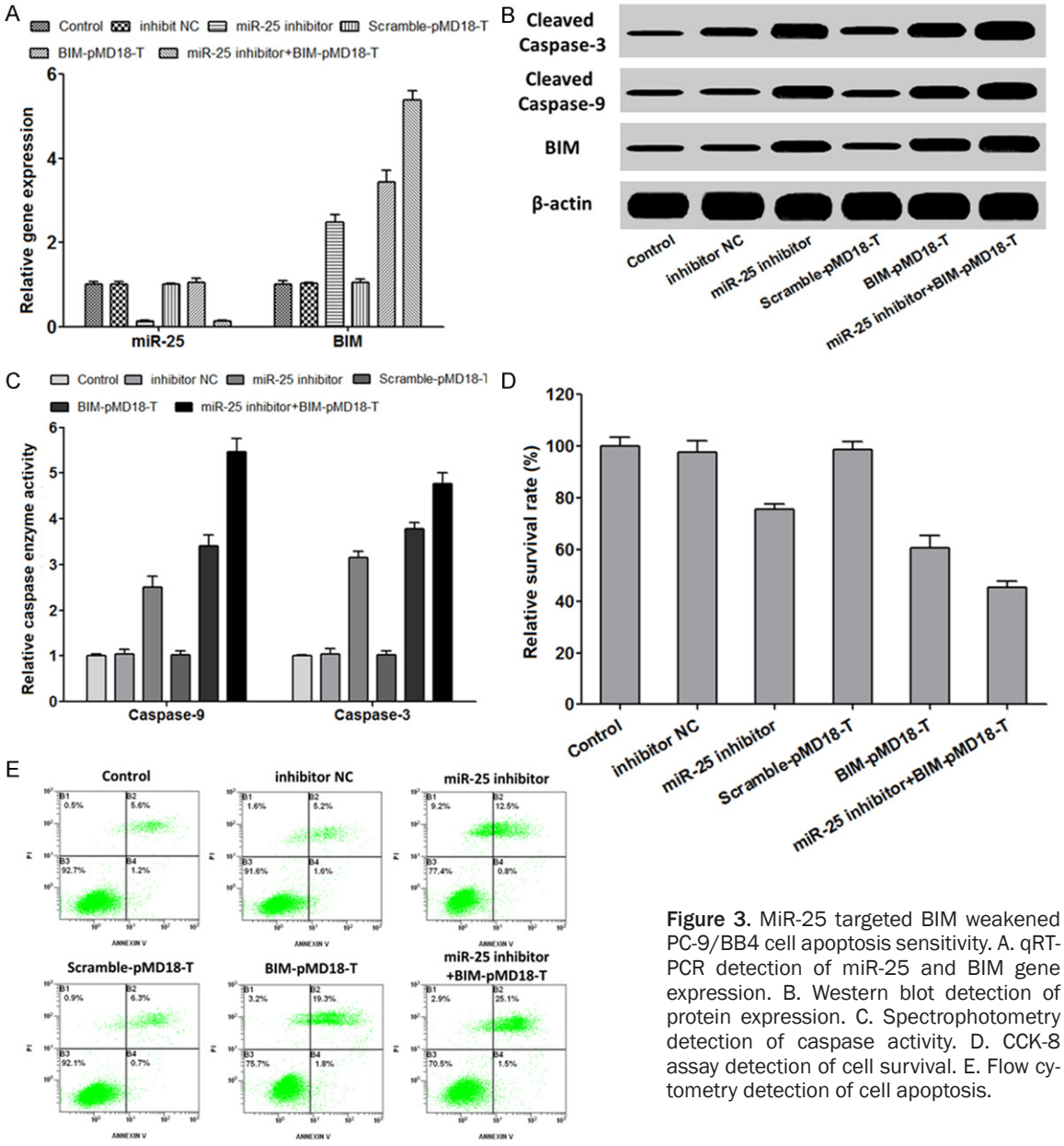

Figure 3. MiR-25 targeted BIM weakened PC-9/BB4 cell apoptosis sensitivity. A. qRT-PCR detection of miR-25 and BIM gene expression. B. Western blot detection of protein expression. C. Spectrophotometry detection of caspase activity. D. CCK-8 assay detection of cell survival. E. Flow cytometry detection of cell apoptosis.

in the last five years [17]. Lung cancer exhibited the leading new onset cases, death cases, and ill cases among malignant tumors in our country [17]. The emergence of new anti-cancer drugs and new treatment measures failed to improve the prognosis of patients significantly. The five-year survival rate of patients in stage I was 60%-80%, and it was 1% in stage IV, resulting in the mean survival rate was 10%-15% [3]. As the main treatment of advanced NSCLC, the efficacy of chemotherapy has reached a plateau, which is difficult to further improve the survival of patients. With the further development of molecular targeted therapy, small molecule TKI gefitinib targeting EGFR obtained significant clinical effect on NSCLC patients in advanced stage [18]. Compared with traditional chemotherapy, EGFR-TKI can effectively extend the progression-free survival time and improve the quality of life, especially on those with EGFR mutations [19]. However, EGFR-TKI drugs induce serious acquired drug resistance, and its effectiveness can only maintain for 6-10 months. Most patients getting curative effects are easy to relapse after remission after a certain time. Almost all the patients exhibit new prog-

ress after a period of remission. Drug resistance has become an important restriction for EGFR-TKI drugs application in clinic.

BIM, a member of the subfamily of Bcl-2 protein family containing BH3-only domain structure, plays an important role in the induction of apoptosis [8, 9]. The BH3-only domain structure of BIM is the main domain to promote apoptosis, which transfers Bax from cytoplasm to the mitochondria and increases the permeability of mitochondrial membrane. It further leads to cytochrome C release to cytoplasm to activate Caspase-9 and downstream Caspase-3, resulting in mitochondrial dependent endogenous apoptosis pathway activation [20]. Sakakibara, *et al*. found that BIM expression in NSCLC tumor tissue significantly declined, suggesting that BIM reduction may be involved in NSCLC pathogenesis [10]. A variety of investigations demonstrated that anti-cancer drugs significantly upregulated BIM expression except inducing lung cancer apoptosis, indicating that BIM played a role in drugs inducing lung cancer apoptosis [11-13]. Li, *et al.* showed that under the induction of gefitinib, BIM expression in drug resistant lung cancer cell line was obviously higher than that in drug sensitive cell line, revealing that BIM expression and function changes may participate in drug resistance formation [14]. Xiang, *et al.* reported that miR-25 expression markedly upregulated in NSCLC tissues [15]. Yang, *et al.* found that miR-25 level in NSCLC tumor tissue was apparently higher than that in para-carcinoma tissue [16], suggesting that miR-25 elevation may promote canceration in NSCLC. MicroRNA target gene prediction presented that miR-25 had good target complementary relationship with the 3'-UTR of BIM. This study investigated the relationship between lung cancer drug resistance and abnormal expression of miR-25 and BIM.

Our results demonstrated that PC-9 cell viability obviously declined after gefitinib treatment on concentration gradient, whereas PC-9/BB4 cells were slighter influenced by gefitinib treatment, confirming PC-9/BB4 cells gained resistance to gefitinib. Certain concentration of gefitinib treatment significantly induced PC-9 cell apoptosis but showed lower sensitivity to PC-9/BB4 cells apoptosis. BIM expression in PC-9/BB4 cells was obviously lower than that in PC-9 cells. On the contrary, miR-25 level in PC-9/BB4 cells was higher. Gefitinib significantly increased BIM expression in drug sensitive cell line PC-9 but not in PC-9/BB4, which was similar with the result published by Li [21]. It suggested that BIM downregulation in PC-9/BB4 cells may be the result caused by miR-25 abnormal elevation, as their dysregulation may play a role in mediating drug resistance. Dual luciferase reporter assay showed that miR-25 mimic and miR-25 inhibitor reduced and upregulated luciferase activity in HEK293 cell lysis, respectively. They also declined and enhanced BIM gene and protein expressions in PC-9/BB4 cells, respectively, confirming that BIM was regulated by miR-25. Thus, we further discussed the function of miR-25 and BIM dysregulation in gefitinib induced NSCLC cell apoptosis and drug resistance. MiR-25 inhibitor transfection and/or BIM overexpression obviously increased BIM expression in PC-9/BB4 cells, enhanced caspase-9 and caspase-3 activities, elevated cell apoptosis and drug sensitivity. Li, *et al*. reported that BIM silencing inhibited gefitinib induced cell apoptosis, which was similar to our results [21]. For the relationship between BIM and EGFR-TKI drug sensitivity, Zhao, *et al*. demonstrated that the PFS (4.7 months) and drug response rate (25%) in NSCLC patients with BIM gene deletion polymorphism were significantly lower than that without deletion polymorphism (PFS at 11 months and drug response rate at 66%) [22]. Huang, *et al.* also found that NSCLC patients with BIM gene polymorphism showed worse prognosis and curative effect of EGFR-TKI drug compared with patients without polymorphism [23]. This study explained the role of BIM in mediating NSCLC cancer cell acquired drug resistance and apoptosis insensitivity and related epigenetic mechanism from the aspect of microRNA. MiR-25 was found to promote NSCLC cell proliferation through targeting FBXW7 [15]. Moreover, Chen, *et al*. proved that miR-25 attenuated NSCLC cell apoptosis by targeting RGS3 [24]. Wu, *et al.* presented that miR-25 suppressed NSCLC cell apoptosis by regulating MOAP1 [25]. In this study, miR-25 abnormal upregulation weakened the apoptosis sensitivity of PC-9/BB4 cells induced by gefitinib through inhibiting BIM expression, explaining the influence of miR-25 on drug resistance and tumor promotion.

MiR-25 upregulated, while BIM declined in gefitinib resistant PC-9/BB4 cells. MiR-25 inhibited

cell apoptosis induced by gefitinib through targeting BIM, which may play a role in PC-9/BB4 cell drug resistance.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yi Tang, The 1st Department of Thoracic Surgery, The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, 283 Tongzipo Road, Changsha 410013, Hunan, P. R. China. Tel: +86-731-88651-900; Fax: +86-731-88651900; E-mail: yitangzxc@sina.com

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