Neoplasma 2025; 72(1–2): 118–127

doi:10.4149/neo_2025_250113N19

EIF4E promotes gefitinib resistance in non-small cell lung cancer by activating the Wnt/β-catenin pathway

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Received January 13, 2025 / Accepted April 15, 2025

Tyrosine kinase inhibitors (TKIs) like gefitinib, which target the epidermal growth factor receptor (EGFR), show considerable therapeutic effectiveness in non-small cell lung cancer (NSCLC) with EGFR-activating mutations. Nevertheless, the resistance that develops against EGFR-TKIs diminishes their therapeutic impact in clinical settings. This investigation focused on the impact of eukaryotic translation initiation factor 4E (eIF4E) on gefitinib resistance in NSCLC. Using the CCK-8 assay, the influence of different gefitinib concentrations on cell proliferation was examined. eIF4E and EGFR expressions were verified by qRT-PCR and/or western blotting in NSCLC cell lines. Moreover, the effects of eIF4E knockdown in gefitinib-treated PC9/GR cells were detected by CCK-8, flow cytometry, colony formation assays, and xenograft tumor model. eIF4E expression was remarkably upregulated in the gefitinib-resistant PC9/GR cells. Moreover, the expression levels of eIF4E in NSCLC cell lines exhibited a dose-dependent increase following gefitinib administration. The function assays demonstrated that reducing eIF4E levels hindered the proliferation of PC9/GR cells and enhanced the apoptosis-inducing effects of gefitinib both in vitro and in vivo, and also had an inhibitory action on the Wnt/β-catenin pathway. Taken together, these results suggested that eIF4E confers gefitinib resistance in NSCLC by regulating the Wnt/β-catenin pathway. Therefore, eIF4E is a possible therapeutic target for improving therapeutic action in NSCLC patients who have developed resistance to gefitinib.

Key words: lung cancer; gefitinib; eIF4E; Wnt; β-catenin

Lung cancer (LC) is the malignant tumor with the highest incidence rate worldwide and ranks first in cancer-related deaths in China [1, 2]. Non-small cell lung cancer (NSCLC) accounts for about 85% of LC, with a 5-year overall survival rate of less than 20%, and an overall 10-year survival rate of less than 10% [3]. Currently, the main treatment for LC is still surgical resection, although new methods such as multimodal therapy and immunotherapy have made rapid progress in recent years, the invasive and metastatic nature of LC has led to a large portion of patients with a poor prognosis [4, 5].

The genetic change with the highest detection rate in NSCLC patients is epidermal growth factor receptor (EGFR) mutation, which leads to a high response to EGFR-tyrosine kinase inhibitors (EGFR-TKIs) [6, 7]. Although EGFR-TKIs contribute significantly to prolonging the progression-free survival of patients and improve the quality of life and tolerability of patients, the emergence of acquired resistance after 10–14 months of EGFR-TKI treatment has become a major obstacle to clinical therapy [8–9]. Gefitinib, a first-generation EGFR-TKI, exhibits antitumor activity through competitive binding to the ATP-binding pocket of the EGFR structural domain and subsequent blockade of downstream proliferative signaling [10]. However, patients treated with gefitinib also developed resistance [11]. Therefore, the search for molecular mechanisms that can overcome gefitinib resistance has become a pressing issue in tumor therapy.

Eukaryotic Initiation Factor-4E (eIF4E), one of the core components of eukaryotic protein translation initiation, plays an important role in the process of protein translation initiation [12]. Current studies have shown that eIF4E is a key molecule in influencing the biological behavior of tumor growth, invasion, and metastasis, and has become a

new target for anti-tumor therapy [13, 14]. Enhanced eIF4E function caused by eIF4E overexpression or activation of the Ras and PI3K/ AKT pathways selectively upregulates the translation of cancer-related genes, including c-Myc, cyclin D1, and Bcl-2, which promotes tumor growth, angiogenesis, and cell survival [15, 16]. In addition, eIF4E knockdown also inhibits the proliferation and angiogenesis [13, 17]. More importantly, enhanced eIF4E expression enhances patient resistance to multiple chemotherapeutic agents [18].

The Wnt/β-catenin signaling pathway has been found to be involved in a variety of biological functions in past studies, including energy metabolism, embryonic development, and tumorigenesis [19, 20]. It is also considered to be an important signaling pathway that promotes cell proliferation and invasion, and tumor growth and metastasis. A study has shown that β-catenin is a target of eIF4E to promote tumor progression [21]. Besides, the eIF4E/β-catenin axis has been shown to maintain leukemia stem cell function [22]. It has been demonstrated that inhibition of eIF4E or β-catenin can effectively inhibit the proliferation, migration, and survival of cervical cancer cells [22]. However, the role of eIF4E-mediated activation of the Wnt/β-catenin signaling pathway in gefitinib resistance in lung cancer remains to be elucidated.

In this study, the PC9 cells and the gefitinib-resistant PC9/GR cells were used to investigate the effects of eIF4E *in vivo* and *in vitro* for gefitinib resistance. The findings suggested that knocking down eIF4E could effectively decrease the expression of the Wnt/β-catenin signaling pathway and inhibit the EGFR activation, subsequently attenuating the gefitinib resistance.

Materials and methods

Cell culture and transfection. The human NSCLC cell lines HCC827, H1299, PC9, and gefitinib-resistant PC9/GR cells were obtained from the iCell Bioscience Inc. (Shanghai, China). All cell lines were grown in RPMI-1640 medium (TransGen Biotech, Beijing, China) supplemented with 10% fetal bovine serum (Opcel, Nei Monggol, China) and maintained in a humidified incubator with 5% CO_2 at 37 °C. Gefitinib (#HY-50895; MedChemExpress) was put into the growth media at a dose of 0.1 μmol/l to maintain the gefitinib resistance property of PC9/GR cells. The small interference RNA (siRNA)-eIF4E#1 (GGGCUCUGUACAACCAUAUTT), siRNA-eIF4E#2 (GGAGGACGAUGGCUAAUUATT), and siRNA-eIF4E#3 (GGUGAUAAGAUAGCAAUAUTT) were obtained from Gene Pharmacy (Shanghai, China). Next, siRNAs and its corresponding negative control (siRNA-NC) were transfected into PC9/GR cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After transfection for 48 h, the cells were collected for quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analyses.

Cell viability assay. The HCC827, H1299, PC9, PC9/GR cells, as well as the transfected PC9/GR cells, were seeded in a 96-well plate (3,000 cells/well) and cultured for 24 h. Then, all cells were challenged with different concentrations of gefitinib (MedChemExpress, USA). After 48 h of treatment, 10 μl of Cell Counting Kit-8 (CCK-8) solution (C0039, Beyotime, China) was added to each well. Finally, the absorbance of each well was detected by a microplate reader at 450 nm.

Cell apoptosis assay. The BD 556547 Annexin V-FITC Apoptosis Detection Kit (Livning, Beijing, China) was selected to assess cell apoptosis of the transfected PC9/GR cells. After 48 h of gefitinib treatment (10 μM) in transfected PC9/GR cells, the cells were incubated with 5 μl Annexin V-FITC and 10 μl PI for 15 min in the dark. Then, the cell apoptosis rate was measured by using flow cytometry (NovoCyte, Agilent Technologies).

Clone formation assay. For clone formation experiments, the transfected PC9/GR cells were cultured in 6-well plates at a density of 1×10^3/well. The RPMI-1640 complete medium containing gefitinib was changed every 3–4 days. After culturing for 14 days, the clones were fixed with 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet for 15 min. Finally, the cell colonies were photographed and counted using a microscope (ICX41, SOPTOP, Ningbo, China).

Animal experiment. Twenty-four 5-to 6-week-old male BALB/c nude mice were obtained from the SLAC LABORATORY ANIMAL CO., LTD (SCXK(Hu)2022-0004, Shanghai, China) and maintained in Hangzhou Hunter Testing Biotechnology Co., Ltd (SYXK(Zhe)2024-0003, Zhejiang, China). For the xenograft tumor assay, the PC9/GR cells transfected with siRNA-eIF4E#1 and siRNA-NC, as well as the PC9 cells, were subcutaneously injected into the region of the axilla of mice, at a density of 1×10^6 cells/100 μl in Matrigel. When the tumor volume reached 50 mm^3, the mice in the gefitinib-treated groups were given 30 mg/kg of gefitinib orally every day for 21 days. The mice in the PC9/GR+siRNA-NC group were treated with the same volume of physiological saline. Also, the tumor size was measured every 3 days using a vernier caliper, and the tumor volume was calculated (V = (tumor width (W)^2 × tumor length (L))/2. After 21 days of treatment, the mice were sacrificed, and the tumor tissues were collected and weighed for immunohistochemistry (IHC) and western blot assays.

The research protocols were approved by the Laboratory animal management and ethics committee of Hangzhou Hunter Testing Biotechnology Co., Ltd (approval number: IACUC/HTYJ-8201-46).

IHC analysis. Tumor tissues were fixed with 4% paraformaldehyde, dehydrated, paraffin-embedded, and sectioned. After antigen retrieval, the 4 μm of sections were incubated with primary antibodies against Ki-67 (1:100, #AF0198, Affinity), phospho-EGFR (Ser1070) (1:100, #AF3044, Affinity), Wnt1 (1:100, #AF5315, Affinity), and β-catenin

(1:100, #AF6266, Affinity) at 4 °C overnight. Thereafter, horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was added and incubated at 37 °C for 30 min. After that, the sections were treated with diaminobenzidine (DAB) and counterstained with hematoxylin. Finally, the pictures were photographed under a microscope.

qRT-PCR assay. Total RNA of NSCLC cell lines was extracted using Universal RNA Purification Kit (#AG21024, Amyjet, Wuhan, China) and reverse-transcribed into cDNA through HiFiScript cDNA Synthesis Kit (#CW2569M, ComWin Biotech Co., Ltd, Beijing, China) according to the instructions. The relative expression levels of EGFR and eIF4E were determined by a LightCycler® 96 real-time PCR instrument (Roche, Switzerland) using SYBR Green Master Mix (Yeasen, Shanghai, China). The primer sequences were synthesized by Sangon (Shanghai, China): EGFR Forward: 5'-CGCAGTTGGGCACTTTTGAA-3'; Reverse: 5'-TTCCAGACAAGCCACTCACC-3'; eIF4E Forward: 5'-CGCACACTTTCTGGAAACCAC-3'; Reverse: 5'-GTGTCTGCGTGGGACTGATA-3'; GAPDH Forward: 5'-GAAGGTGAAGGTCGGAGTC-3'; Reverse: 5'-GAAGATGGTGATGGGATTTC-3'. At last, the results were analyzed using the $2^{-\Delta\Delta CT}$ method.

Western blot assay. Total proteins of NSCLC cell lines and tumor tissues were collected using RIPA buffer consisting of protease inhibitors. Subsequently, the cell supernatant was quantified by a bicinchoninic acid (BCA) kit (#P0012, Beyotime, China). After denaturation, an equal amount of protein samples was separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% skimmed milk for 1.5 h at room temperature, the membranes were cultivated with primary antibodies against eIF4E (1:1000, #AF6110, Affinity), EGFR (1:1000, #ab52894, Abcam), Wnt1 (1:1000, #AF5315, Affinity), β-catenin (1:1000, #8480T, Cell Signaling Technology), p-EGFR (1:1000, #AF3044, Affinity), Bax (1:1000, #AF0120, Affinity), Bcl-2 (1:2000, #ab182858, Abcam), Cleaved Caspase-3 (1:1000, #AF7022, Affinity), and GAPDH (1:10000, #10494-1-A, Proteintech) overnight at 4 °C. After washing 3 times with TBST, the membranes were incubated with the indicated secondary antibodies for 1 h at room temperature. Finally, the protein bands were visualized using a chemiluminescent (ECL) reagent (Beyotime, China) and analyzed by ImageJ software.

Statistical analysis. The results were represented as mean ± SD. Student's t-test was used to estimate the differences between the two groups, while the one-way analysis of variance (ANOVA) was selected for comparing multiple groups using GraphPad software (version 8.0, USA). A p-value <0.05 was determined statistically significant.

Results

Cytotoxicity of gefitinib to NSCLC cell lines. To assess the effect of gefitinib on NSCLC cell lines, the cytotoxicity of gefitinib on HCC827, H1299, PC9, and PC9/GR cells was detected by CCK-8 experiments. The result found that the cell viability of HCC827, H1299, PC9, and PC9/GR cells was evidently decreased in a dose-dependent manner (Figures 1A–1D). The IC50 values of gefitinib on HCC827,

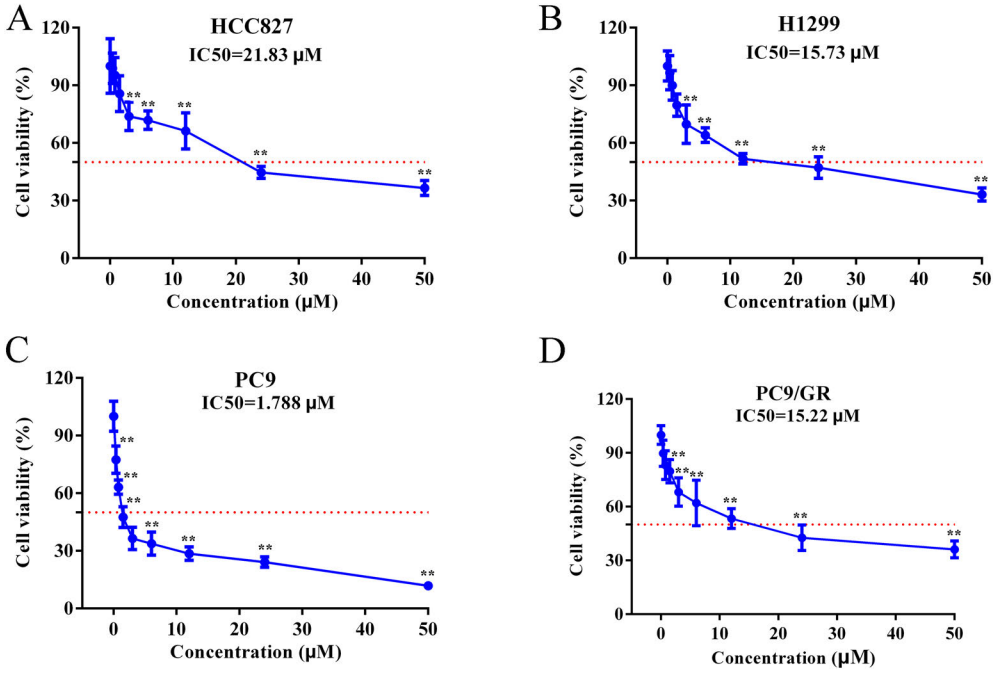

Figure 1. Sensitivity of LC cells to gefitinib. Inhibitory effects on cell viability of A) HCC827, B) H1299, C) PC9, and D) PC9/GR cells were measured by CCK-8 assays. **p<0.01 compared with control (0 μM) group

H1299, PC9, and PC9/GR cells are 21.83, 15.73, 1.78, and 15.22 μM at 48h, respectively.

eIF4E was remarkably upregulated in NSCLC cell lines. To explore the effect of eIF4E in gefitinib resistance of NSCLC, we first assessed the expression levels of eIF4E in normal BEAS-2B cells and NSCLC cell lines HCC827, H1299, PC9, and PC9/GR cells. As expected, the mRNA and protein expressions of eIF4E and EGFR in NSCLC cell lines were observably higher than those in control cells (Figures 2A, 2B), especially in PC9/GR cells. These data showed that the eIF4E levels might be associated with the changes in EGFR and help in the gefitinib resistance in NSCLC cells. To explore whether eIF4E contributes to the acquired resistance of NSCLC cells to EGFR-TKIs, the HCC827, H1299, PC9, and PC9/GR cells were treated with gefitinib at different concentrations (0, 3, 6, 12, 24 μmol/l). Western blot assays suggested that gefitinib

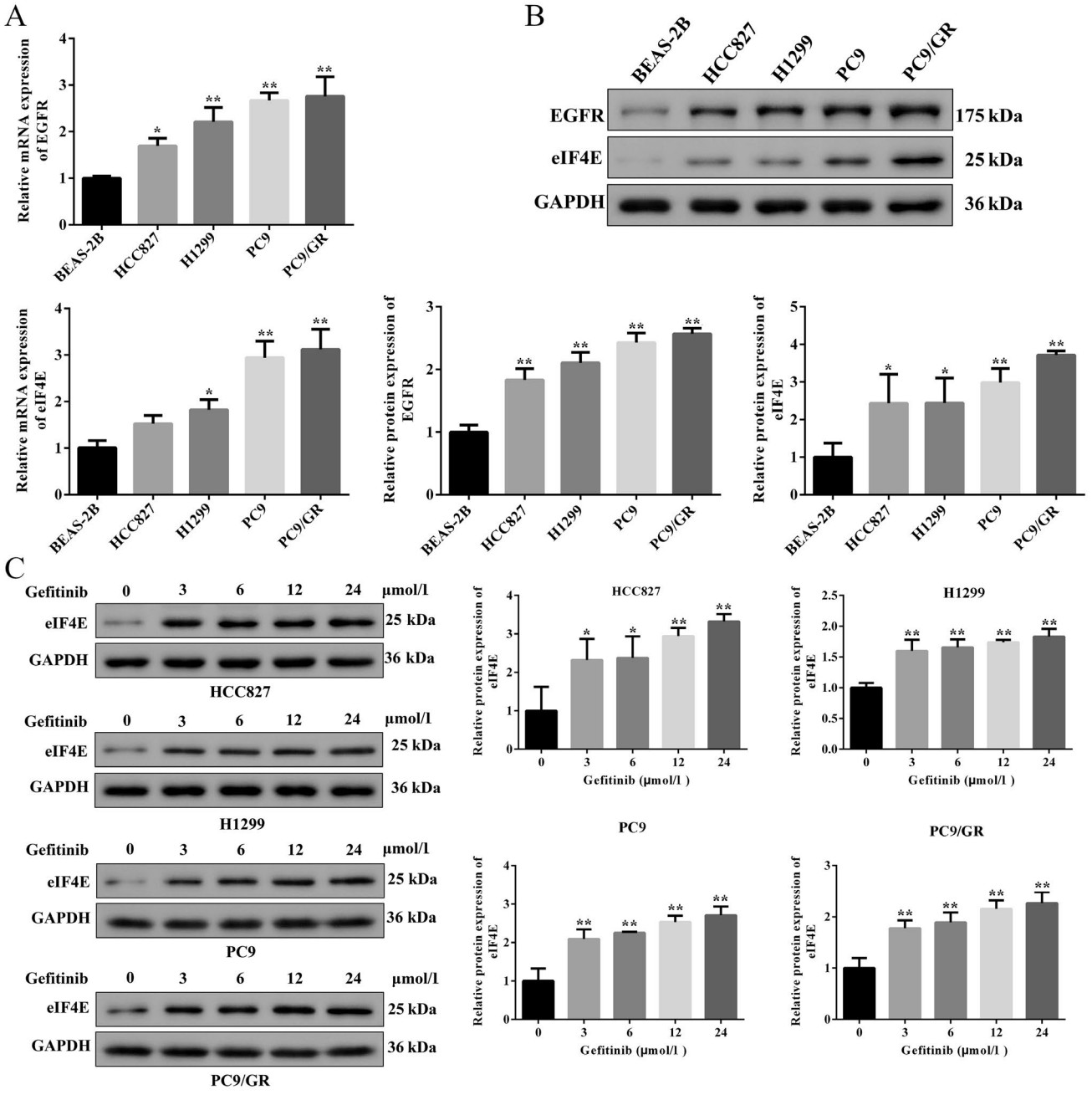

Figure 2. EGFR and eIF4E expressions were increased in gefitinib-resistant LC cells. A) EGFR and eIF4E mRNA levels of LC cells were analyzed by RT-qPCR. B) Protein expression levels of EGFR and eIF4E of LC cells were analyzed by western blot assays. C) Protein expression levels of eIF4E of LC cells treated with different concentrations of gefitinib were determined by western blotting. *p<0.05, **p<0.01 compared with control (0 μM) group

could promote the protein expression of eIF4E in NSCLC cell lines in a dose-dependent manner (Figure 2C). Collectively, these results suggested that eIF4E may be related to the acquired resistance of gefitinib in NSCLC with activation of EGFR.

eIF4E deletion recovers the sensitivity of gefitinib in PC9/GR cells. To further investigate the biological function of eIF4E in gefitinib-resistant PC9/GR cells, we used siRNAs to knock down eIF4E expression in PC9/GR cells. The qPCR and western blot results revealed that siRNA-eIF4E#1 remarkably downregulated the expression levels of eIF4E (Figures 3A, 3B). Additionally, we also used the different concentrations (0, 3, 6, 12, 24 μmol/l) of gefitinib to treat the PC9/GR cells with eIF4E knockdown. CCK-8 analysis showed that knockdown of eIF4E significantly downregulated cell viability in PC9/GR cells compared with negative-transfected PC9/GR cells (Figure 3C). Furthermore, flow cytometry and colony formation assays found that deleting eIF4E could significantly promote cell apoptosis and impair cell proliferation in PC9/GR cells upon gefitinib treatment (Figures 3D, 3E). Taken together, these data suggested that eIF4E deletion can promote the cytotoxicity of gefitinib in PC9/GR cells *in vitro*.

eIF4E deletion attenuated Wnt/β-catenin pathway and p-EGFR in PC9/GR cells. Previous research has shown that the activation of the Wnt/β-catenin pathway is involved in the multidrug resistance in NSCLC [23, 24]. In this study, the roles of eIF4E deletion on expression levels of the Wnt/β-catenin pathway and p-EGFR in gefitinib-treated PC9/GR cells were estimated using western blot assay. As shown in

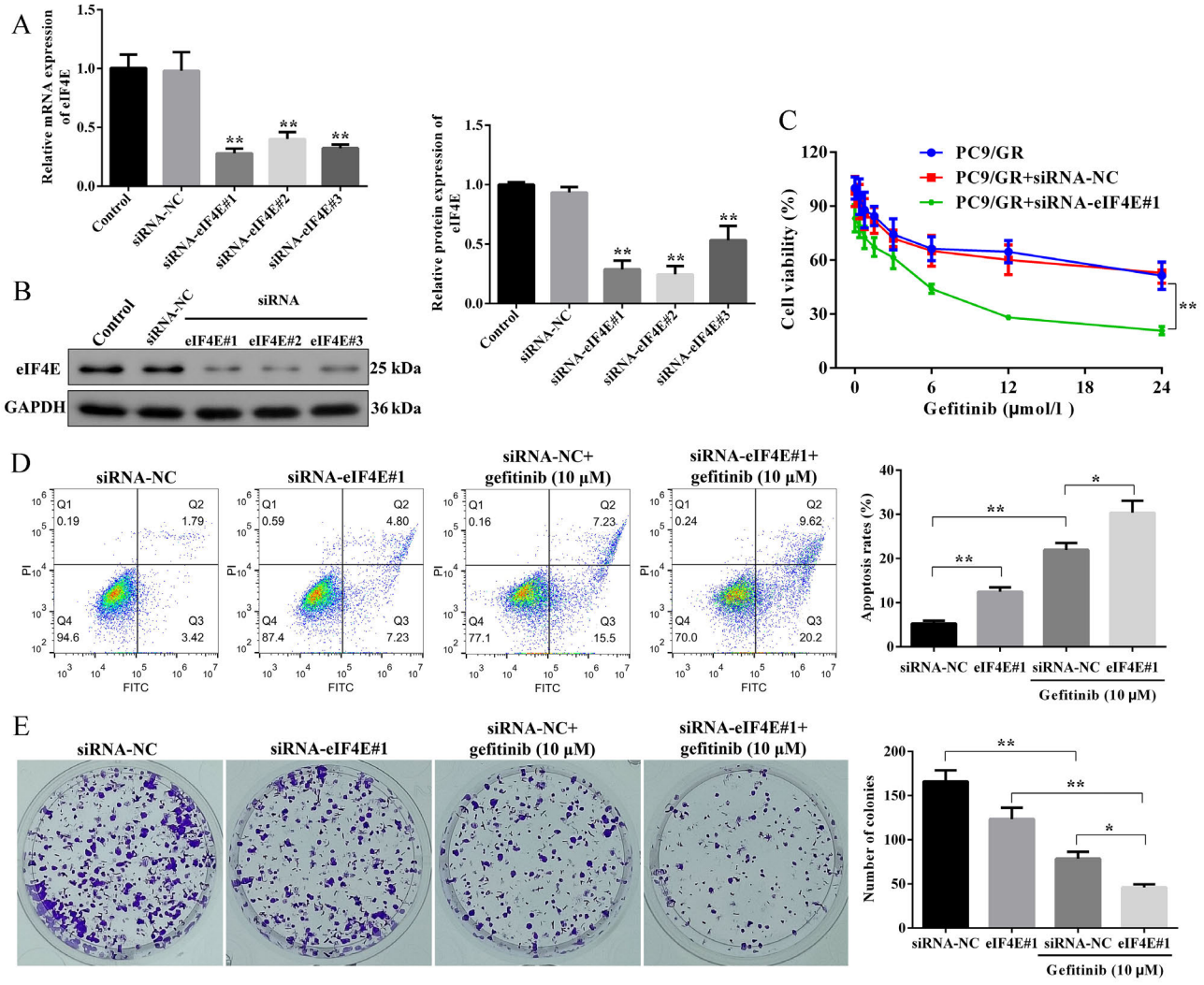

Figure 3. Knockdown of eIF4E improves the sensitivity of PC9/GR cells to gefitinib. A) RT-qPCR and B) western blot assays were used to detect eIF4E expression in PC9/GR cells transfected with si-eIF4E. C–E) Cell proliferation ability and apoptosis of PC9/GR cells were assessed by CCK-8, flow cytometry, and colony formation assays, respectively, with eIF4E knockdown and gefitinib treatment. *p<0.05, **p<0.01 compared with control or siRNA-NC group

Figure 4A, the protein expression levels of Wnt1, β-catenin, and p-EGFR in PC9 cells were dose-dependently decreased after gefitinib therapy, while a slight reduction in the expression levels of Wnt1, β-catenin, and p-EGFR was found in PC9/GR cells. Subsequently, the changes of the Wnt/β-catenin pathway and p-EGFR in gefitinib-treated PC9/GR cells with eIF4E deletion were determined. The results showed that an outstanding decrease in the expression of Wnt1, β-catenin, and p-EGFR when gefitinib dosages gradually increased was observed in PC9/GR+siRNA-eIF4E#1 cells (Figure 4B). Therefore, eIF4E may be an essential Wnt/β-catenin pathway and p-EGFR-related factor to intervene in the acquired resistance of gefitinib in PC9/GR cells.

eIF4E deletion promoted tumor-suppressive effects of gefitinib *in vivo.* We further investigate the effect of eIF4E knockdown on tumor growth in PC9/GR-bearing mice treated with gefitinib. And, the mice implanted with PC9 cells followed by gefitinib treatment served as a negative control, while the PC9/GR+siRNA-NC group was a positive control. Interestingly, the results showed that the knockdown of eIF4E could significantly inhibit the tumor growth of the mice in the gefitinib+PC9/GR+siRNA-eIF4E#1 group compared with the gefitinib+PC9/GR+siRNA-NC group (Figures 5A–5D). Additionally, Ki-67, p-EGFR, Wnt1, β-catenin, and Bcl-2 levels were decreased, and Bax and Caspase-3 levels were increased in the tumor tissues in the gefitinib+PC9 and gefitinib+PC9/GR+siRNA-eIF4E#1 groups (Figures 5E, 5F). Taken together, these results suggested that eIF4E inhibition restrained the tumor growth and promoted sensitivity to gefitinib in nude mice by inhibiting the Wnt/β-catenin and p-EGFR pathways.

Discussion

Targeted treatments, such as EGFR-TKIs like gefitinib, sevortinib, topotecanib, and camartinib, have emerged as the preferred first-line therapy for patients with NSCLC. However, despite their impressive effectiveness, the challenge of drug resistance remains an inevitable issue. The classification of TKI resistance includes two main categories: intrinsic and acquired [25]. The most prevalent mechanism for acquired resistance to first-generation reversible EGFR-TKIs involves mutations in the T790M region of the ATP-binding pocket of EGFR, which diminishes the effectiveness of ATP-competitive EGFR-TKIs [26]. Therefore, it is important to clarify the specific mechanism of EGFR-TKI resistance.

eIF4E is recognized for its role in initiating translation as a protein that binds to the 5' cap of mRNA [28]. It is also found to be upregulated in various types of human cancers and is linked to aggressive tumor behaviors [27]. Zhang et al. have demonstrated that inhibition of MAP kinase interacting serine/threonine kinase (MNK)/eIF4E could improve anlotinib resistance in NSCLC [28]. Similarly, eIF4E deletion also promoted chemotherapy sensitivity in gastric cancer [29], breast cancer [30], cervical cancer [31], and hepatocellular carcinoma [32]. Nonetheless, no research has concentrated on the role of eIF4E in gefitinib-acquired resistance in NSCLC. In this study, the CCK-8 assay showed that the IC50 of PC9/GR cells treated with gefitinib significantly increased compared to the corresponding parental cells. Nonetheless, the IC50 values are markedly different from those reported in the study conducted by Xu et al. [33]. It is plausible to hypothesize that this could be attributed to factors related to the experimental techniques, such as the heterogeneity among the cells or the length of gefitinib treatment. Furthermore, Xu et al. reported that the phosphorylation levels of eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1) and eIF4E were significantly increased in both the PC9/GR and HCC827/GR cells compared to the PC9 and HCC827 cells [33]. Similarly, we found that gefitinib promoted eIF4E expression levels in NSCLC cell lines in a dose-dependent manner, including the PC9/GR cells. Additionally, the results showed that knockdown of eIF4E attenuated cell viability and increased cell apoptosis in PC9/GR cells treated with gefitinib *in vitro* and *in vivo*. Consistent with our results, Li and colleagues revealed that there was a significant increase in eIF4E expression in human NSCLC tissues relative to normal tissues, and eIF4E knockdown decreased c-Met expression and partially reestablished cellular responsiveness to erlotinib [34]. Thus, these data also suggest that the eIF4E may play a significant role in the proliferation of NSCLC cells and their resistance to apoptosis, thereby contributing to the development of acquired resistance to gefitinib therapy; however, the precise underlying mechanisms remain to be elucidated.

To elucidate the molecular mechanism by which eIF4E facilitates resistance to chemotherapy, we concentrated on signaling cascades that are integral to the preservation of EGFR activation. It is known that the interplay and transactivation between the EGFR and Wnt/β-catenin signaling pathways may play a significant role in the enhanced malignancy of neoplasms [35], and recent investigations have underscored the critical role of the Wnt/β-catenin signaling cascade in mediating the drug resistance mechanisms in NSCLC [36, 37]. For example, Jia et al. found that PFTK1 (also named CDK14) engages with LRP6 and stimulates Wnt/β-catenin signaling, thereby reducing gefitinib-induced cell apoptosis [38]. Moreover, we found that the knockdown of eIF4E reduced the expression levels of Wnt1 and β-catenin, as well as the phosphorylation of EGFR in gefitinib-treated PC9/GR cells and tumor-bearing mice. In addition, it has been noted that the induction of apoptosis in NSCLC cells occurs through the suppression of Wnt/β-catenin and EGFR signaling pathways and the activation of Caspase-3 cleavage, independent of the EGFR mutation status [39]. Consistently, our findings demonstrated that the administration of gefitinib, coupled with the depletion of eIF4E, enhanced apoptotic processes *in vivo* by elevating the levels of Bax and cleaved Caspase-3. Importantly, a prior study has established that eIF4E overexpression enhances the translation of

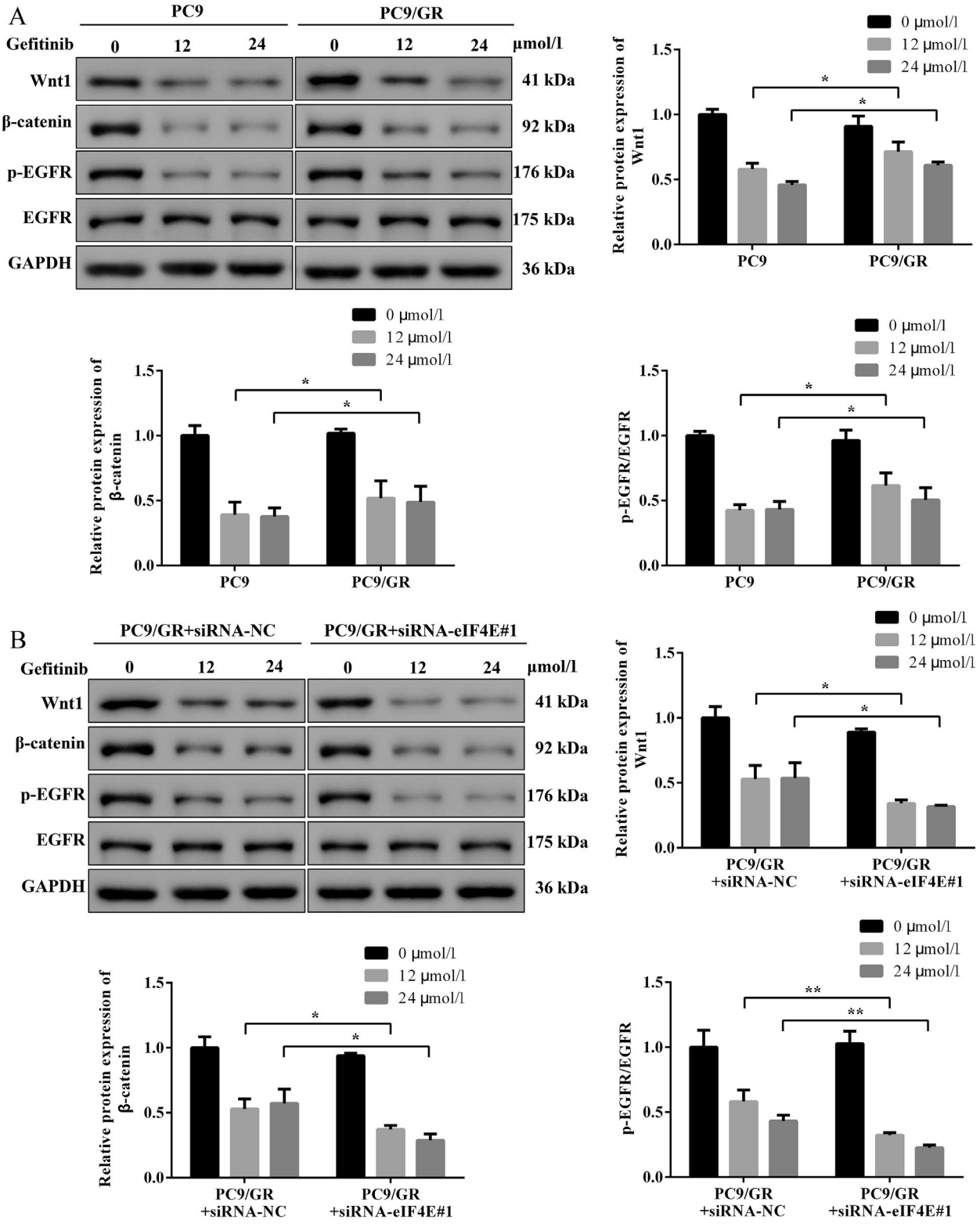

Figure 4. Effect of eIF4E knockdown on Wnt/β-catenin pathway in gefitinib-treated PC9/GR cells. A) PC9/GR and PC9 cells were exposed to various doses of gefitinib (0, 12, 24 μmol/l) for 24h. Western blotting was carried out to investigate the expression levels of Wnt1, β-catenin, phosphorylated (p)-EGFR, and EGFR. B) The PC9/GR cells transfected with si-eIF4E were pretreated with various doses of gefitinib (0, 12, 24 μmol/l) for 24 h. Protein expression levels of Wnt1, β-catenin, p-EGFR, and EGFR in PC9/GR cells, as determined using western blotting. *p<0.05, **p<0.01

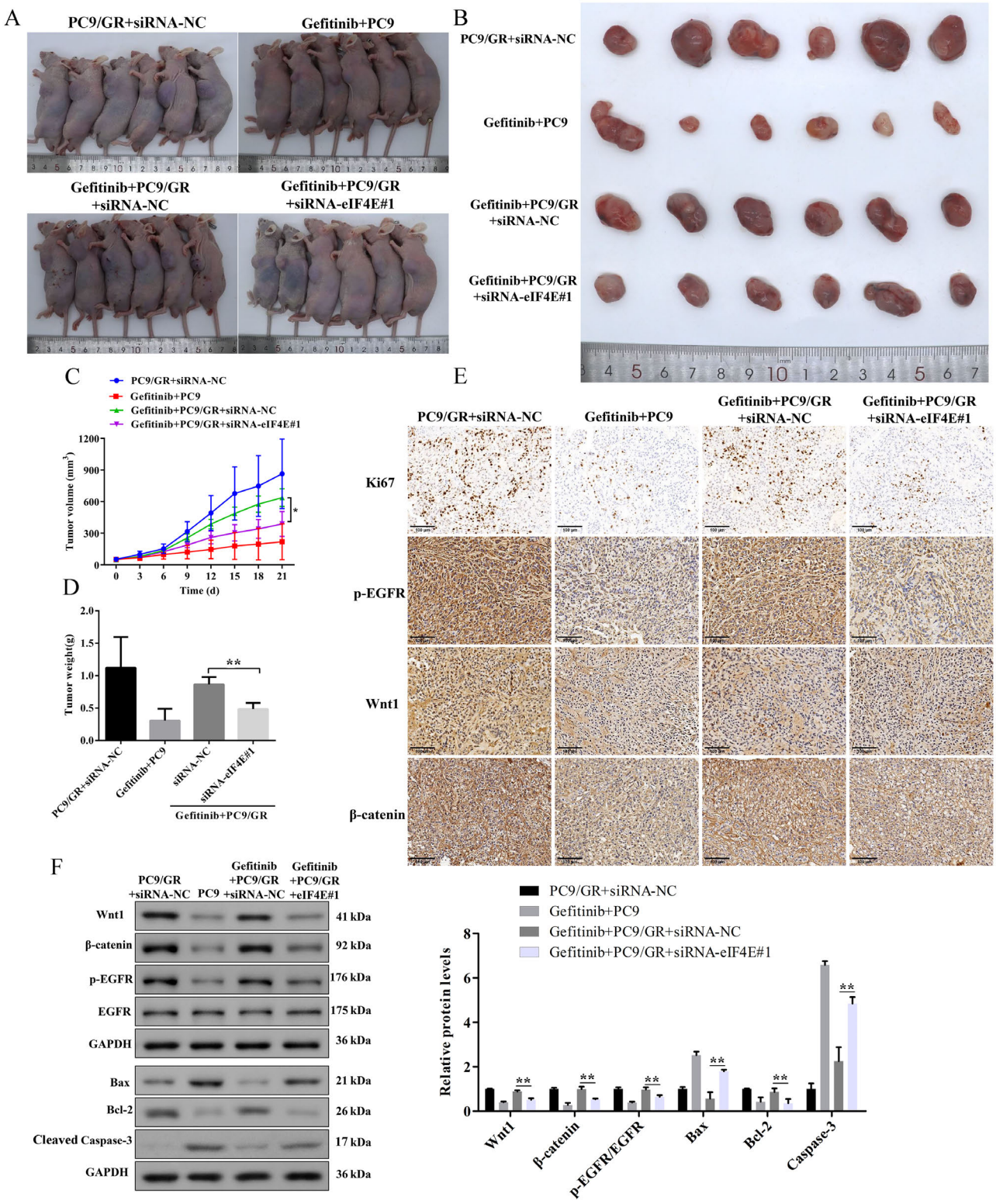

Figure 5. Knockdown of eIF4E increases the sensitivity of PC9/GR cells to gefitinib in nude mice. A, B) Representative images of xenograft tumors in gefitinib-treated nude mice injected with PC9/GR cells with eIF4E knockdown. C) The volume of the xenograft tumors. D) The weight of the xenograft tumors. E) Representative images of immunohistochemical staining of Ki-67, p-EGFR, Wnt1, and β-catenin in xenograft tumors. Scale bar = 100 μm. F) Western blot assays were used to assess the expression levels of Wnt1, β-catenin, p-EGFR, EGFR, Bax, Bcl-2, and Caspase-3 of xenograft tumors. **p<0.01

oncogenic mRNAs, including those encoding Wnt/β-catenin [40]. Therefore, we hypothesize that the eIF4E-mediated resistance to gefitinib is, at least in part, contingent upon the activation of the Wnt/β-catenin signaling pathway, necessitating further investigation into the involvement of other related signaling cascades.

In conclusion, our results demonstrate the effect of eIF4E on the development of gefitinib resistance in NSCLC. Also, these findings suggest that eIF4E may serve as a novel therapeutic target for NSCLC exhibiting gefitinib resistance through modulation of the Wnt/β-catenin signaling pathway. However, there are certain limitations in our study that need to be mentioned. First, the effect of eIF4E overexpression on the expression of Wnt/β-catenin and EGFR pathways in NSCLC cell lines upon gefitinib treatment remains unclear. Second, although gefitinib can increase eIF4E under certain conditions, we did not further investigate the mechanism associated with EIF4EBP1 that interacts with eIF4E.

Acknowledgments: This study was supported by the Program of Taizhou Science and Technology (No.20ywa05).

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