

# Krüppel-like factor 4 promotes *c-Met* amplification-mediated gefitinib resistance in non-small-cell lung cancer

Wei Feng<sup>1</sup>  | Qianyi Xie<sup>1</sup> | Suo Liu<sup>1</sup> | Ying Ji<sup>1</sup> | Chunyun Li<sup>2</sup> | Chunle Wang<sup>3</sup> | Longyu Jin<sup>1</sup>

<sup>1</sup>Departments of Cardiothoracic Surgery, Third Xiangya Hospital of Central South University, Changsha, China

<sup>2</sup>Departments of Pediatrics, Third Xiangya Hospital of Central South University, Changsha, China

<sup>3</sup>Department of Cardiac Surgery, Second Xiangya Hospital of Central South University, Changsha, China

## Correspondence

Longyu Jin, Department of Cardiothoracic Surgery, Third Xiangya Hospital of Central South University, Changsha City, Hunan, China.

Email: jinlongyu1123@163.com

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Gefitinib has been widely used in the first-line treatment of advanced *EGFR*-mutated non-small-cell lung cancer (NSCLC). However, many NSCLC patients will acquire resistance to gefitinib after 9-14 months of treatment. This study revealed that Krüppel-like factor 4 (*KLF4*) contributes to the formation of gefitinib resistance in *c-Met*-overexpressing NSCLC cells. We observed that *KLF4* was overexpressed in *c-Met*-overexpressing NSCLC cells and tissues. Knockdown of *KLF4* increased tumorigenic properties in gefitinib-resistant NSCLC cell lines without *c-Met* overexpression, but it reduced tumorigenic properties and increased gefitinib sensitivity in gefitinib-resistant NSCLC cells with *c-Met* overexpression, whereas overexpression of *KLF4* reduced gefitinib sensitivity in gefitinib-sensitive NSCLC cells. Furthermore, Western blot analysis revealed that *KLF4* contributed to the formation of gefitinib resistance in *c-Met*-overexpressing NSCLC cells by inhibiting the expression of apoptosis-related proteins under gefitinib treatment and activating the *c-Met*/Akt signaling pathway by decreasing the inhibition of  $\beta$ -catenin on phosphorylation of *c-Met* to prevent blockade by gefitinib. In summary, this study's results suggest that *KLF4* is a promising candidate molecular target for both prevention and therapy of NSCLC with *c-Met* overexpression.

## KEYWORDS

Akt, *c-Met*, gefitinib resistance, *KLF4*, NSCLC

## 1 | INTRODUCTION

Lung cancer is a primary cause of cancer-related deaths worldwide, and non-small-cell lung cancer (NSCLC) is the prevalent pathological type of lung cancer, accounting for approximately 85% of cases.<sup>1</sup> Although the pathogenesis of lung cancer is complex, genetic susceptibility variants are a major cause.<sup>1,2</sup> The most prominent genetic factor associated with the responsiveness to NSCLC treatment is a

mutation in *EGFR*,<sup>3</sup> which can activate the downstream PI3K/Akt/mTOR and KRas/Raf/Mek/Erk pathways to promote the malignant progression of NSCLC.<sup>4</sup> At present, gefitinib, a first-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), shows strong therapeutic effects against NSCLC with an activating *EGFR* mutation.<sup>4,5</sup> However, many NSCLC patients will acquire resistance to gefitinib after a median of 9-14 months of treatment.<sup>6</sup> The T790M of *EGFR* is the most common EGFR-TKI resistance mechanism, which confers resistance by increasing the ATP affinity.<sup>7</sup> Furthermore, *c-Met* amplification is a frequently reported mechanism of acquired resistance to EGFR-TKI and has been reported in approximately 20% of NSCLC cases following EGFR-TKI treatment.<sup>8,9</sup> *c-Met*

**Abbreviations:** BAX, BCL2-associated X; CoIP, co-immunoprecipitation; EGFR, epidermal growth factor receptor; ERBB3, Erb-B2 receptor tyrosine kinase 3; HGF, hepatocyte growth factor; hTERT, human telomerase reverse transcriptase; *KLF4*, Krüppel-like factor 4; NSCLC, non-small-cell lung cancer; TKI, tyrosine kinase inhibitor.

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amplification causes EGFR-TKI resistance because *c-Met* amplification can increase the expression of *c-Met* protein which can activate the Erb-B2 receptor tyrosine kinase 3 (ERBB3)/PI3K/Akt signaling pathway, and this pathway is also the downstream signaling pathway of EGFR.<sup>10</sup>

Krüppel-like factor 4 (*KLF4*), as a transcription factor, is expressed in the epithelium of a variety of tissues<sup>11</sup> and has multiple roles in physiological and disease processes, such as cellular processes, vascular functions, and tumorigenesis.<sup>11,12</sup> However, current studies have reported the dual nature of *KLF4* as either an oncogene or a tumor suppressor gene with an unclear mechanism in tumorigenesis.<sup>12,13</sup> *KLF4* functions as a tumor suppressor gene to suppress the proliferation, invasion, and metastasis of tumor cells in kidney cancer,<sup>14</sup> gastric cancer,<sup>15</sup> and prostate cancer,<sup>16</sup> but it was identified as an oncogene in breast cancer.<sup>17</sup> Paradoxically, *KLF4* functions as both an oncogene and tumor suppressor gene in colon cancer<sup>18,19</sup> and skin cancer.<sup>20,21</sup> Several studies have shown that tumor tissues had lower *KLF4* levels compared with normal adjacent tissues.<sup>22-24</sup> *KLF4* might function as a tumor suppressor gene to suppress lung cancer growth by inhibiting human telomerase reverse transcriptase (hTERT) and MAPK signaling.<sup>22</sup> *KLF4* deletion can promote lung cancer formation and progression by activating the mutated oncogene *KRAS*.<sup>23</sup> Another study revealed that activation of oncogenic *c-Met* could increase *KLF4* expression in glioblastoma cells and glioblastoma stem cells.<sup>25,26</sup>

The present study aimed to examine what role *KLF4* plays in *c-Met* amplification-mediated gefitinib-resistant NSCLC patients and elucidate the underlying molecular mechanisms to provide a theoretical basis for molecule inhibitors targeting transcription factors and protein kinases as antitumor therapy.

## 2 | MATERIALS AND METHODS

### 2.1 | Tissue collection and ethics statement

Eighteen primary NSCLC patients undergoing tumor resection were recruited at the Third Xiangya Hospital of Central South University (Changsha, China) from June 2016 to December 2016. None of the patients had received gefitinib treatment, chemotherapy, or radiotherapy before surgery. Appropriate ethical approval was obtained from the Third Xiangya Hospital Ethics Committee, and written informed consent was obtained from all patients. Fresh NSCLC tumor tissues and their adjacent non-malignant lung tissues were sampled and stored at  $-80^{\circ}\text{C}$ .

### 2.2 | *c-Met* genomic amplification assay

Genomic DNA was extracted from NSCLC cells and resected tumor tissues using a MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa) following the manufacturer's protocol. Quantitative PCR was carried out to analyze *c-Met* genomic amplification in extracted DNA samples using QuantiTect SYBR Green PCR Kits (Qiagen). Primers used for *c-Met* were (5'- to 3'-) F-

ATCAACATGGCTCTAGTTGTC and R-GGGAGAATATGCAGT-GAACC.<sup>27</sup> Data were analyzed by relative quantitation using the  $\Delta\Delta\text{Ct}$  method.<sup>27</sup> A value  $>2$  was considered as the *c-Met* genomic amplification.

### 2.3 | Chemicals and cell lines

Gefitinib was obtained from Selleck Chemicals (Houston, TX, USA). Epidermal growth factor was obtained from Peprotech (Rocky Hill, NJ, USA). Cell lines from ATCC (Gaithersburg, MD, USA) were cultured in DMEM (293T), Eagle's minimum essential medium (MRC5), or RPMI-1640 (A549, H460, H1299, H1975, H1993, HCC4006, and HCC827) containing 10% FBS. We generated the gefitinib-resistant HCC827 cells (HCC827GR) from the gefitinib-sensitive HCC827 cells by exposing it to increasing concentrations of gefitinib for 6 months.<sup>28</sup>

### 2.4 | Lentiviral infection

Lentivirus packaging was carried out as previously described.<sup>29</sup> Briefly, 293T cells were co-transfected with an appropriate proportion (4:3:1) of the lentivirus plasmids (p $\text{tko.1-sh-KLF4}$ , p $\text{tko.1-sh-}\beta$ -Catenin, p $\text{HBLV-Flag-c-Met}$ , p $\text{HBLV-HA-KLF4}$ , p $\text{HBLV-KLF4}$ , and p $\text{HBLV-}\beta$ -Catenin), packaging plasmids psPAX2 and envelope plasmid pMD2.G. The supernatant containing lentivirus was collected at 48-72 hours post-transfection followed by infection into different NSCLC cell lines. At 24 hours after lentivirus infection, all cells were cultured for another 6 days in the medium containing 1-2  $\mu\text{g/mL}$  puromycin (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.5 | Cell proliferation assay

Cell proliferation was assessed using MTS and clonogenic assays. For the MTS assay, stably transfected NSCLC cell lines were seeded ( $2.5 \times 10^3$  cells/well in 200  $\mu\text{L}$ ) into 96-well plates and divided into the gefitinib group (four wells) and the control group (four wells). The cells were later treated with gefitinib (1  $\mu\text{mol/L}$ ) or vehicle (DMSO) according to their respective groups. After incubation for an additional 0, 24, 48, or 72 hours, 20  $\mu\text{L}$  MTS reagents (Promega, Madison, WI, USA) were added to each well. The absorbance at 490 nm of each well was measured on a spectrophotometer after incubation for 1 hour. For the clonogenic assay, stably transfected NSCLC cell lines were seeded ( $1 \times 10^3$  cells/well) in a 6-well plate and divided into the gefitinib and control groups and then treated with gefitinib (1  $\mu\text{mol/L}$ ) or vehicle (DMSO). After 1-2 weeks of culture, colonies were stained with crystal violet and analyzed using Image-Pro Plus 7.0 software.

### 2.6 | Cell apoptosis analysis

Stably transfected NSCLC cell lines ( $2 \times 10^5$  cells/well) were seeded into 6-well plates and divided into the gefitinib group (three wells) and a control group (one well). After incubation for 24, 48, or 72 hours, cells in the gefitinib group were treated with 1  $\mu\text{mol/L}$  gefitinib. After incubation for 96 hours, all cells were collected and

washed twice with cold PBS. Cells were then stained using the annexin-V-FITC/propidium iodide apoptosis kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Stained cells were detected using Becton-Dickinson FACSverse (BD Biosciences). The data were analyzed using FlowJo version 10 software. Another parallel experiment was conducted for Western blot analysis.

## 2.7 | Western blot analysis

Cell or tissue samples were separately harvested and lysed by RIPA buffer (CWbio, Beijing, China) containing 0.1 mg/mL PMSF (Keygen, Nanjing, China), protease inhibitor, and Phospho-stop (Roche, Mannheim, Germany). Protein aliquots (30  $\mu$ g) were separated by 10% SDS-PAGE and transferred to 0.45- $\mu$ m PVDF membranes (Millipore, Billerica, MA, USA). The blots were blocked for 1 hour at room temperature and incubated separately with primary antibodies (diluted 1:1000) against EGFR, p-EGFR<sup>Tyr1068</sup>, c-Met, p-c-Met<sup>Tyr1234/1235</sup>, Akt, p-Akt<sup>Ser473</sup>, p-Akt<sup>Thr308</sup>, Erk, p-Erk<sup>Thr202/Tyr204</sup>, cleaved poly (ADP-ribose) polymerase, cleaved caspase3 (Cell Signaling Technology, Boston, MA, USA), hTERT, hepatocyte growth factor (HGF), KLF4 (Abcam, Cambridge, USA), BCL2-associated X (BAX),  $\beta$ -catenin, and GAPDH (Proteintech, Wuhan, China) overnight at 4°C. The PVDF membranes were washed with TBS-Tween 20 and then incubated separately with appropriate HRP-conjugated secondary antibodies (diluted 1:5000) (Cell Signaling Technology) for 1 hour at room temperature. After rinsing, the signal on the PVDF membrane was detected by the enhanced chemiluminescence method. The relative protein expression was presented as the ratio of target protein band intensity to GAPDH band intensity using ImageJ software (NIH, Bethesda, MD, USA).

## 2.8 | In vivo tumor growth

Male nude mice, 4-6 weeks old, were obtained from the Center for Medical Experiments of Third Xiangya Hospital of Central South University. The research protocol was approved, and the mice were maintained according to the Institutional Guidelines of the Animal Ethics Committee of Central South University. Nude mice were randomized into four different groups (12 mice/group) and inoculated separately with HCC827GR-sh-Mock, HCC827GR-sh-KLF4, HCC827-Control, or HCC827-KLF4 lung cancer cells ( $4 \times 10^6$  cells/100  $\mu$ L) in the left axilla. When all tumors reached a mean volume of 50 mm<sup>3</sup>, the nude mice in each group were randomized into two different subgroups (6 mice/subgroup) and treated with gefitinib (50 mg/kg/day) or vehicle (0.5% Tween-80) for 3 weeks by oral gavage, as previously described.<sup>30</sup> The tumor length and width of each mouse were measured weekly by a digital caliper. The tumor volumes (V) were calculated using the following formula:  $V = \text{length} \times \text{width}^2 / 2$ . The relative tumor volume (RTV) was calculated by the following formula:  $RTV = V_w / V_0$ , in which  $V_w$  represents the volume each week, and  $V_0$  is the initial tumor volume at the beginning of gefitinib treatment. All nude mice were killed 3 weeks after the initial gefitinib treatment, and the tumor tissues were collected for analysis.

## 2.9 | Statistical analysis

Results were analyzed using SPSS version 18 statistical software (IBM SPSS, Chicago, IL, USA). Normally distributed continuous variables were compared using ANOVA or least significant difference t-test, as appropriate. The statistical results are expressed as the mean  $\pm$  SD of three independent experiments. A probability level of  $P < .05$  was considered statistically significant.

## 3 | RESULTS

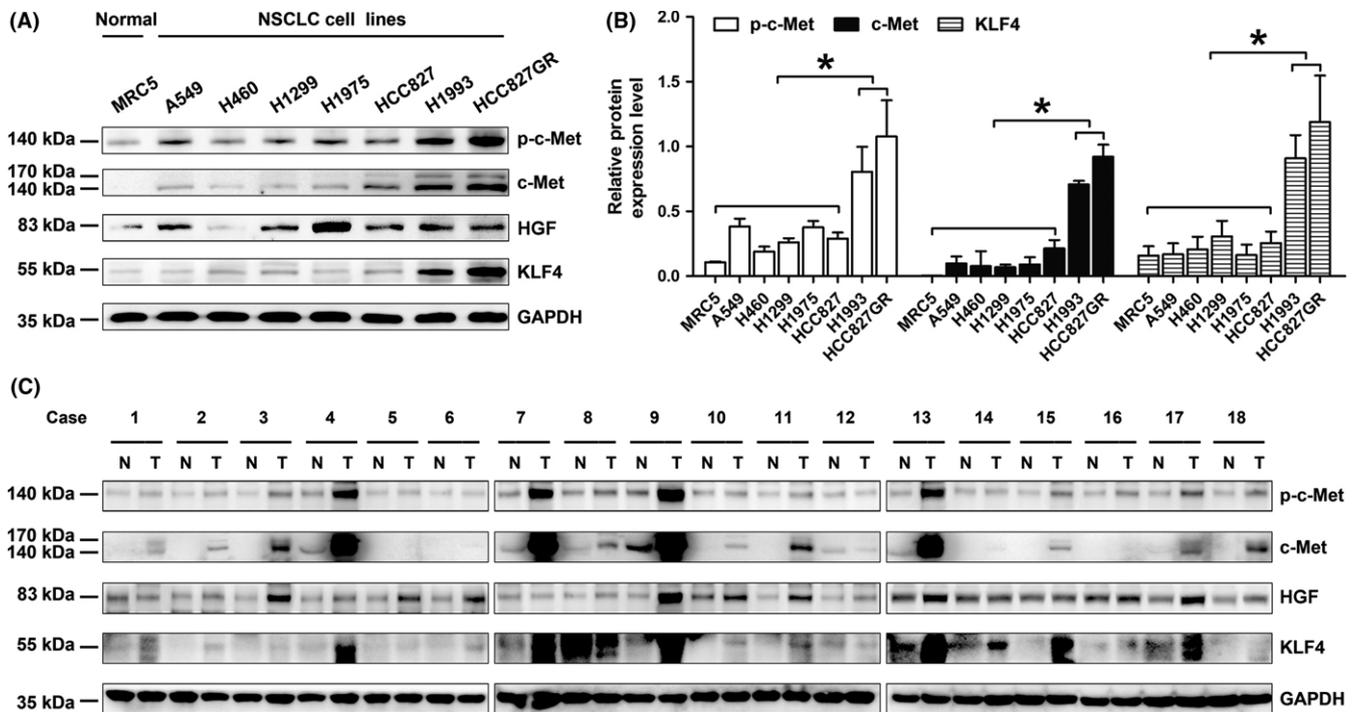
### 3.1 | Overexpression of KLF4 in NSCLC cells with c-Met overexpression

To study the function of KLF4 in gefitinib-resistant NSCLC cells, we adopted normal MRC5 lung cells, a gefitinib-sensitive cell line, and gefitinib-resistant NSCLC cell lines with different mechanisms of resistance for the analyses. These gefitinib-resistant NSCLC cell lines have the most common mechanisms of gefitinib resistance, including EGFR mutation, KRAS mutation, and c-Met amplification, and the sequencing results of these genes are showed in Table 1. Western blot analysis was used to examine the protein expression of p-c-Met, c-Met, HGF, and KLF4 in these cell lines. The results showed that p-c-Met and KLF4 levels were upregulated in the c-Met overexpressing NSCLC cells H1993 and HCC827GR compared with the other cell lines (Figure 1A,B;  $n = 3$ ,  $P < .05$ ). These results are consistent with previous studies, which reported that c-Met activation increased KLF4 expression in in vitro experiments.<sup>25,31</sup> In addition, no significant correlation between KLF4 and HGF was found in Figure 1. We also investigated the expression of p-c-Met, c-Met, HGF, and KLF4 in 18 NSCLC tumor tissues and their adjacent non-malignant lung tissues and their gene information showed in Table 2. The results also showed that p-c-Met and KLF4 were overexpressed in c-Met-overexpressing NSCLC patient samples (Figure 1C; cases 4, 7, 9, and 13). These results indicated that KLF4 is overexpressed in NSCLC cells with c-Met overexpression and might promote the resistance of these cells to gefitinib.

**TABLE 1** EGFR/Ras/c-Met status and tyrosine kinase inhibitor (TKI) sensitivity of different non-small-cell lung cancer (NSCLC) cell lines

NSCLC cell line	EGFR/Ras/c-Met/status	TKI sensitivity
A549	WT/KRasG12V/WT	Resistant
H460	WT/KRasQ61H/WT	Resistant
H1299	WT/WT/WT	Resistant
H1975	L858R + T790M/WT/WT	Resistant
H1993	WT/WT/c-Met amplification	Resistant
HCC4006	Ex19 del/WT/WT	Sensitive
HCC827	Ex19 del/WT/WT	Sensitive
HCC827GR	Ex19 del/WT/c-Met amplification	Resistant

Ex19 del, in-frame deletion in exon 19; KRasG12V, G12V point mutation in exon 2; KRasQ61H, Q61H point mutation in exon 3; L858R, L858R point mutation in exon 21; T790M, T790M point mutation in exon 20.



**FIGURE 1** Krüppel-like factor 4 (KLF4) is overexpressed in non-small-cell lung cancer (NSCLC) cells with c-Met overexpression. A, Western blot analysis revealed that KLF4 was overexpressed in gefitinib-resistant NSCLC cell lines with c-Met overexpression. B, Relative protein expression of p-c-Met, c-Met, and KLF4 in NSCLC cell lines. C, Western blot analysis of tumor tissues (T) and adjacent non-malignant lung tissues (N) showed that KLF4 was overexpressed in NSCLC patients expressing high levels of c-Met (cases 4, 7, 9, and 13). Statistical results are represented as the mean  $\pm$  SD ( $n = 3$ ). \*Significant difference ( $P < .05$ ) in H1993 or HCC827GR vs other cell lines. HGF, hepatocyte growth factor

### 3.2 | Knockdown of *KLF4* increases tumorigenic properties in NSCLC cells without c-Met overexpression, but it reduces tumorigenic properties and increases gefitinib sensitivity in NSCLC cells with c-Met overexpression

Based on the above results, we hypothesized that KLF4 affects gefitinib sensitivity in NSCLC. Therefore, we generated stable *KLF4* knockdown NSCLC cell lines and measured the effect of gefitinib on cell proliferation. The results revealed that knockdown of *KLF4* promoted cell proliferation, including clonogenic ability and cell growth of A549, H460, H1299, and H1975 cells, but did not change their gefitinib sensitivity (Figure S1;  $n = 3$ ,  $P < .05$ ). This tumor-suppressive function of KLF4 might be attributable to negatively regulated hTERT expression in these NSCLC cell lines (Figure S1, right panel) as previous reported.<sup>22</sup> However, knockdown of *KLF4* significantly inhibited cell proliferation of H1993 (Figure 2A) and HCC827GR (Figure 2B) cells ( $n = 3$ ,  $P < .05$ ), and these proliferation properties were further inhibited by treatment with 1  $\mu\text{mol/L}$  gefitinib (Figure 2A,B;  $n = 3$ ,  $P < .05$ ). Furthermore, the results of in vivo experiments confirmed that xenograft growth of HCC827GR cells was also significantly inhibited by knocking down *KLF4* expression, and gefitinib significantly inhibited xenograft growth of *KLF4* knockdown HCC827GR cells after 3 weeks of treatment compared with vehicle treatment (Figure 2C,D;  $n = 6$ ,  $P < .05$ ). These results suggest that

*KLF4* functions as an oncogene in c-Met-overexpressing NSCLC cells and increases their gefitinib resistance.

### 3.3 | Overexpression of *KLF4* reduces tumorigenic properties and gefitinib sensitivity in gefitinib-sensitive NSCLC cells

To further confirm the above conclusion, we generated stable HCC4006 and HCC827 cell lines overexpressing *KLF4* and measured the tumorigenic properties and gefitinib sensitivity using the same methods. The results revealed that overexpression of *KLF4* significantly rescued the gefitinib-induced inhibition of clonogenic ability (Figure 3A,B, left and middle panels;  $n = 3$ ,  $P < .05$ ) and cell growth (Figure 3A,B, right panels;  $n = 3$ ,  $P < .05$ ) of HCC4006 and HCC827 cells. Overexpression of *KLF4* inhibited the proliferation of HCC4006 and HCC827 cells (Figure 3A,B;  $n = 3$ ,  $P < .05$ ) and this tumor-suppressive function might be attributable to inhibited hTERT expression (Figure 3A,B, right panels). To observe this phenomenon in vivo, we produced another nude mouse xenograft model injected with HCC827-Control or HCC827-*KLF4*. We observed that overexpression of *KLF4* significantly inhibited the tumor growth and reduced the gefitinib sensitivity of HCC827 cells (Figure 3B,C;  $n = 6$ ,  $P < .05$ ). These results suggest that *KLF4* functions as a tumor-suppressor gene in gefitinib-sensitive HCC827 cells but reduces their gefitinib sensitivity.

**TABLE 2** *EGFR/c-Met* status of tissue samples from patients with non-small-cell lung cancer

Patient	Specimen	Pathological type	<i>EGFR</i> status	<i>c-Met</i> status
1	Tumor tissue	Squamous cell carcinoma	WT	WT
2	Tumor tissue	Squamous cell carcinoma	WT	WT
3	Tumor tissue	Adenocarcinoma	Ex19 del	WT
4	Tumor tissue	Adenocarcinoma	WT	WT
5	Tumor tissue	Adenocarcinoma	Ex19 del	WT
6	Tumor tissue	Squamous cell carcinoma	WT	WT
7	Tumor tissue	Adenocarcinoma	T790M	<i>c-Met</i> amplification
8	Tumor tissue	Adenocarcinoma	L858R + T790M	WT
9	Tumor tissue	Adenocarcinoma	WT	<i>c-Met</i> amplification
10	Tumor tissue	Adenocarcinoma	L858R + T790M	WT
11	Tumor tissue	Squamous cell carcinoma	WT	WT
12	Tumor tissue	Squamous cell carcinoma	WT	WT
13	Tumor tissue	Squamous cell carcinoma	WT	<i>c-Met</i> amplification
14	Tumor tissue	Adenocarcinoma	L858R	WT
15	Tumor tissue	Adenocarcinoma	Ex19 del	WT
16	Tumor tissue	Adenocarcinoma	WT	WT
17	Tumor tissue	Squamous cell carcinoma	WT	WT
18	Tumor tissue	Adenocarcinoma	Ex19 del	WT

Ex19 del, in-frame deletion in exon 19; L858R, L858R point mutation in exon 21; T790M, T790M point mutation in exon 20.

### 3.4 | Krüppel-like factor 4 regulates gefitinib-induced cell apoptosis in HCC827GR and HCC827 cell lines

To further study the function of KLF4 in the mechanism of gefitinib resistance, we undertook flow cytometry and Western blot analyses to assess the apoptosis status of *KLF4*-knockdown HCC827GR cells and *KLF4*-overexpressing HCC827 cells following treatment with 1  $\mu\text{mol/L}$  gefitinib (Figure 4). The results showed that knockdown of *KLF4* increased the gefitinib-induced apoptosis degree of HCC827GR cells (Figure 4A,C;  $n = 3$ ,  $P < .05$ ). In contrast, overexpression of *KLF4* could reduce the gefitinib-induced apoptosis degree in HCC827 cells under the same gefitinib treatment conditions (Figure 4B,D;  $n = 3$ ,  $P < .05$ ). We carried out the same flow cytometry analysis on four additional gefitinib-resistant NSCLC cell lines, which have the most common mechanisms of gefitinib resistance that differ from those of HCC827GR and H1993 cells. The results showed that knockdown of *KLF4* had no effect on the apoptosis rate of these NSCLC cells after gefitinib treatment (Figure S2). These results indicated that *KLF4* increased the gefitinib resistance of *c-Met*-overexpressing NSCLC cells by inhibiting gefitinib-induced apoptosis.

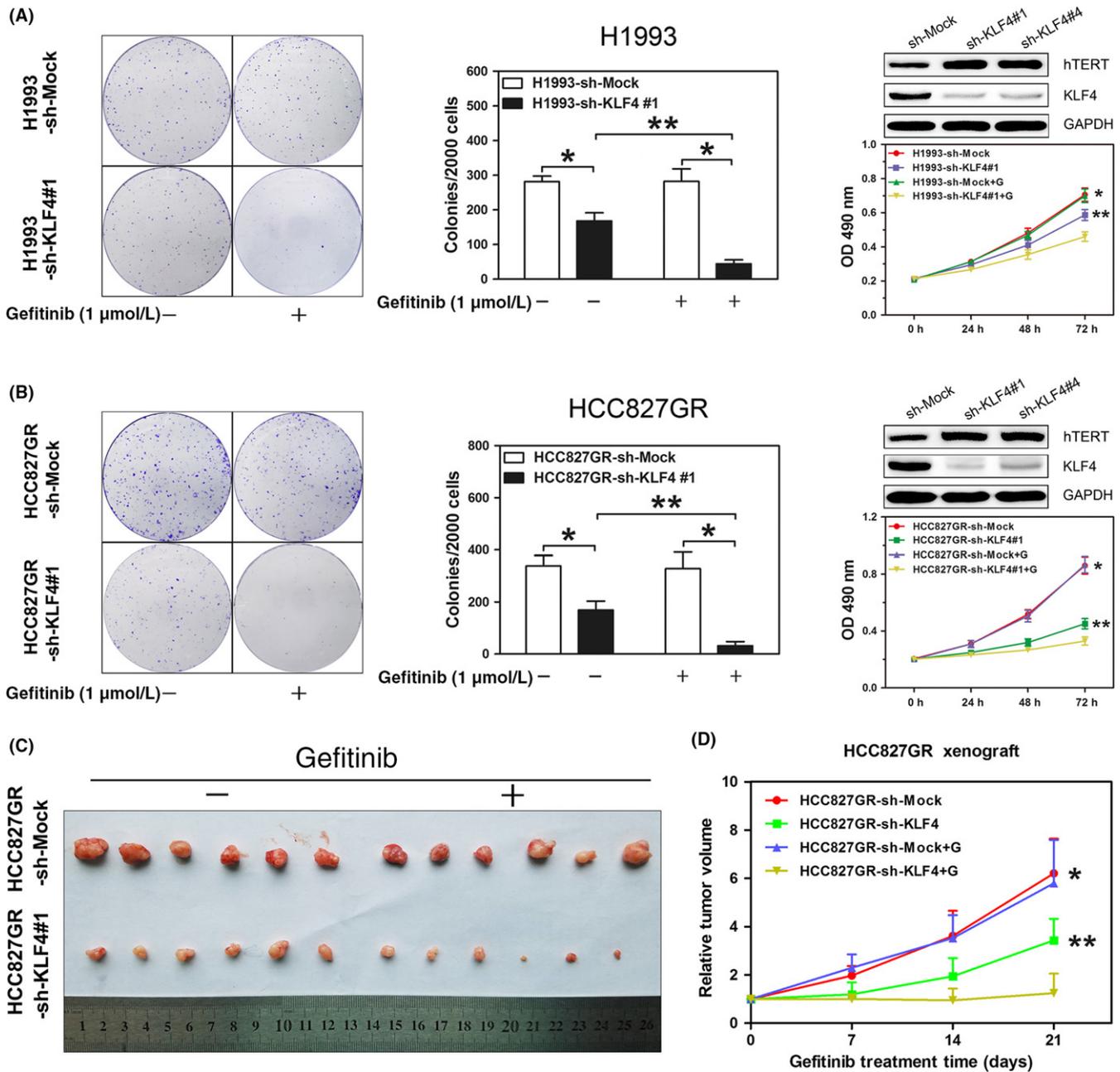
### 3.5 | Krüppel-like factor 4

Regulates the *c-Met*/Akt signaling pathway in HCC827GR and HCC827 cell lines. Previous results confirmed that *KLF4* was a critical factor in the formation of gefitinib resistance in *c-Met*-overexpressing NSCLC cells, but the mechanism was unclear. Consequently, we investigated the function of *KLF4* in the *EGFR*

and *c-Met* pathways of NSCLC cell lines. The results showed that the expression levels of p-*EGFR*, p-*ERK1/2*, total *EGFR*, *c-Met*, Akt, and *Erk1/2* were not altered by *KLF4* in NSCLC cells (Figure 5). However, knocking down *KLF4* significantly inhibited EGF-induced phosphorylation of *c-Met* and its downstream kinase Akt in HCC827GR cells (Figures 5A,S3A;  $n = 3$ ,  $P < .05$ ), and overexpression of *KLF4* significantly promoted EGF-induced phosphorylation of *c-Met* and Akt in HCC827 cells (Figures 5B,S3B;  $n = 3$ ,  $P < .05$ ). In contrast, after gefitinib treatment, knockdown of *KLF4* increased gefitinib-induced inactivation of *c-Met* and Akt in HCC827GR cells (Figures 5C,E,S3C,3E;  $n = 3$ ,  $P < .05$ ), but overexpression of *KLF4* significantly rescued gefitinib-induced inactivation of *c-Met* and Akt in HCC827 cells (Figures 5D,F,S3D,F;  $n = 3$ ,  $P < .05$ ). Furthermore, *KLF4* did not change the phosphorylation of *EGFR*, *c-Met*, Akt, or *Erk* in A549 and H1975 cell lines (Figure S4). These results indicated that *KLF4* promoted the development of gefitinib resistance by activating the *c-Met*/Akt signaling pathway. However, the exact molecular mechanisms are unclear.

### 3.6 | Krüppel-like factor 4 represses the expression of $\beta$ -catenin and inhibits binding between *c-Met* and $\beta$ -catenin

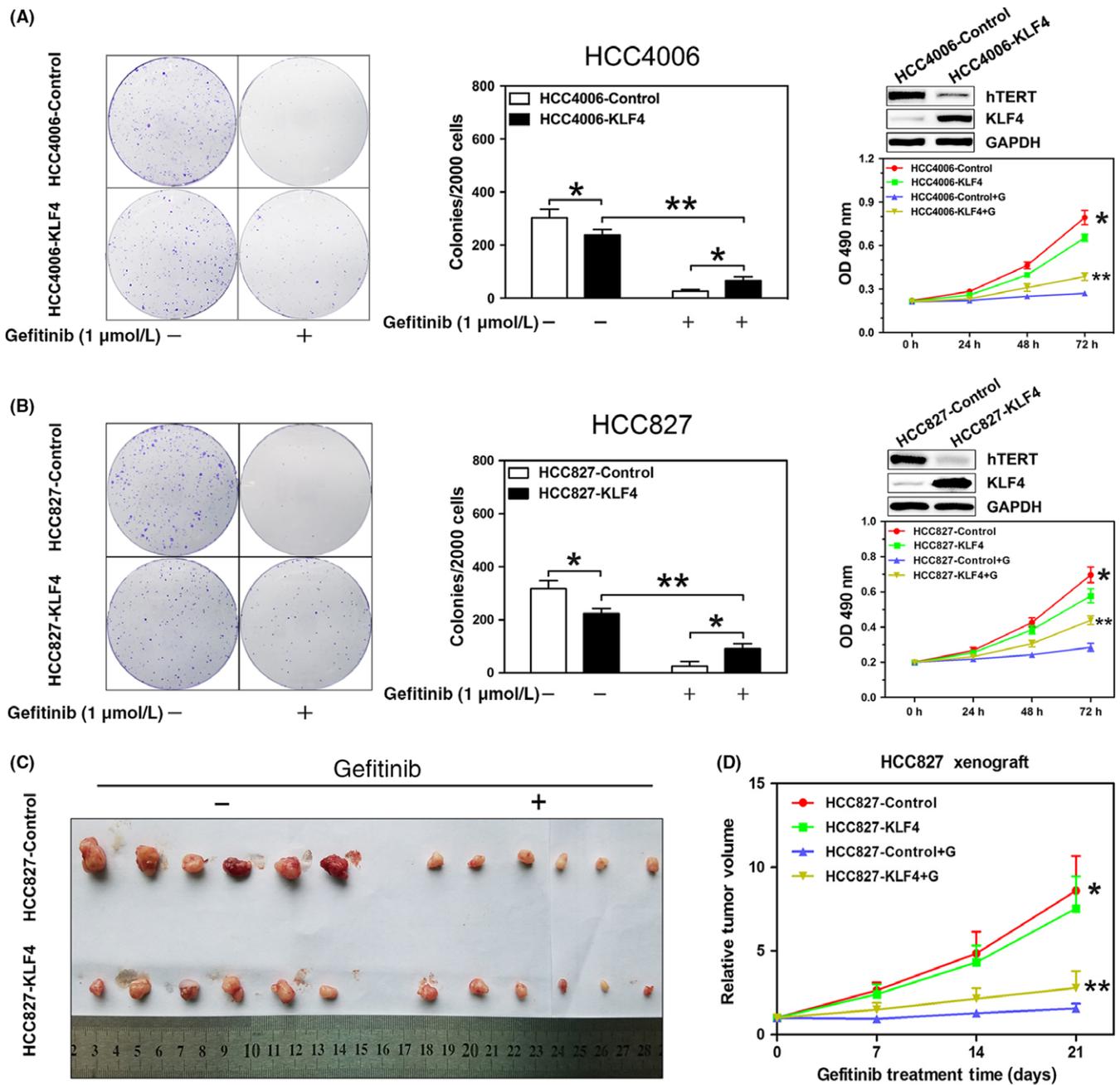
In order to study the molecular mechanism of *KLF4* regulation of *met*, we undertook immunofluorescence and co-immunoprecipitation (CoIP) assays. The methods of immunofluorescence and CoIP are showed in supporting file Data S1. The results showed that *c-Met* was not directly activated by *KLF4* (Figure 6A,B). As a transcription factor, *KLF4* represses the expression of mesenchymal gene  $\beta$ -



**FIGURE 2** Knockdown of Krüppel-like factor 4 (KLF4) in c-Met-overexpressing non-small-cell lung cancer cells reduces tumorigenic properties and increases gefitinib sensitivity. A,B, Clonogenic assays (left and middle panels) and MTS assays (right) were carried out to measure proliferation of KLF4 knockdown (sh-KLF4) and control (sh-Mock) groups of H1993 and HCC827GR cells treated with gefitinib (1  $\mu\text{mol/L}$ ) or vehicle (DMSO). Knockdown of KLF4 increased the inhibition of gefitinib on the proliferation of H1993 and HCC827GR cells; statistical results are represented as the mean  $\pm$  SD ( $n = 3$ ). C,D, Representative photographs of nude mice from each group inoculated with HCC827GR-sh-Mock or HCC827GR-sh-KLF4 cells after 3 wk of treatment with gefitinib (50 mg/kg/d) or vehicle (0.5% Tween-80); knockdown of KLF4 reduced tumorigenic properties of HCC827GR cells but increased their gefitinib sensitivity. Tumors dissected from each group (C) and the relative tumor volume curves of nude mice in each group (D) are shown. Statistical results are represented as mean  $\pm$  SD ( $n = 6$ ). \*sh-KLF4 group vs sh-Mock group,  $P < .05$ . \*\*Treatment with gefitinib (+G) vs vehicle in the sh-KLF4 groups,  $P < .05$ . hTERT, human telomerase reverse transcriptase

catenin (*Cttnb1*) by targeting its promoter.<sup>32</sup> Our results showed that overexpression of KLF4 downregulated the expression of  $\beta$ -catenin (Figure 6B). Furthermore, CoIP results showed that both KLF4 and c-Met interact with  $\beta$ -catenin, but KLF4 inhibited binding between c-Met and  $\beta$ -catenin (Figure 6B, right panel). Further study

showed that overexpression of  $\beta$ -catenin inhibited phosphorylation of c-Met, whereas knockdown of  $\beta$ -catenin augmented its phosphorylation (Figure 6C). This dephosphorylation of c-Met by  $\beta$ -catenin could be explained by the interaction between  $\beta$ -catenin and c-Met, which inhibited phosphorylation of c-Met.

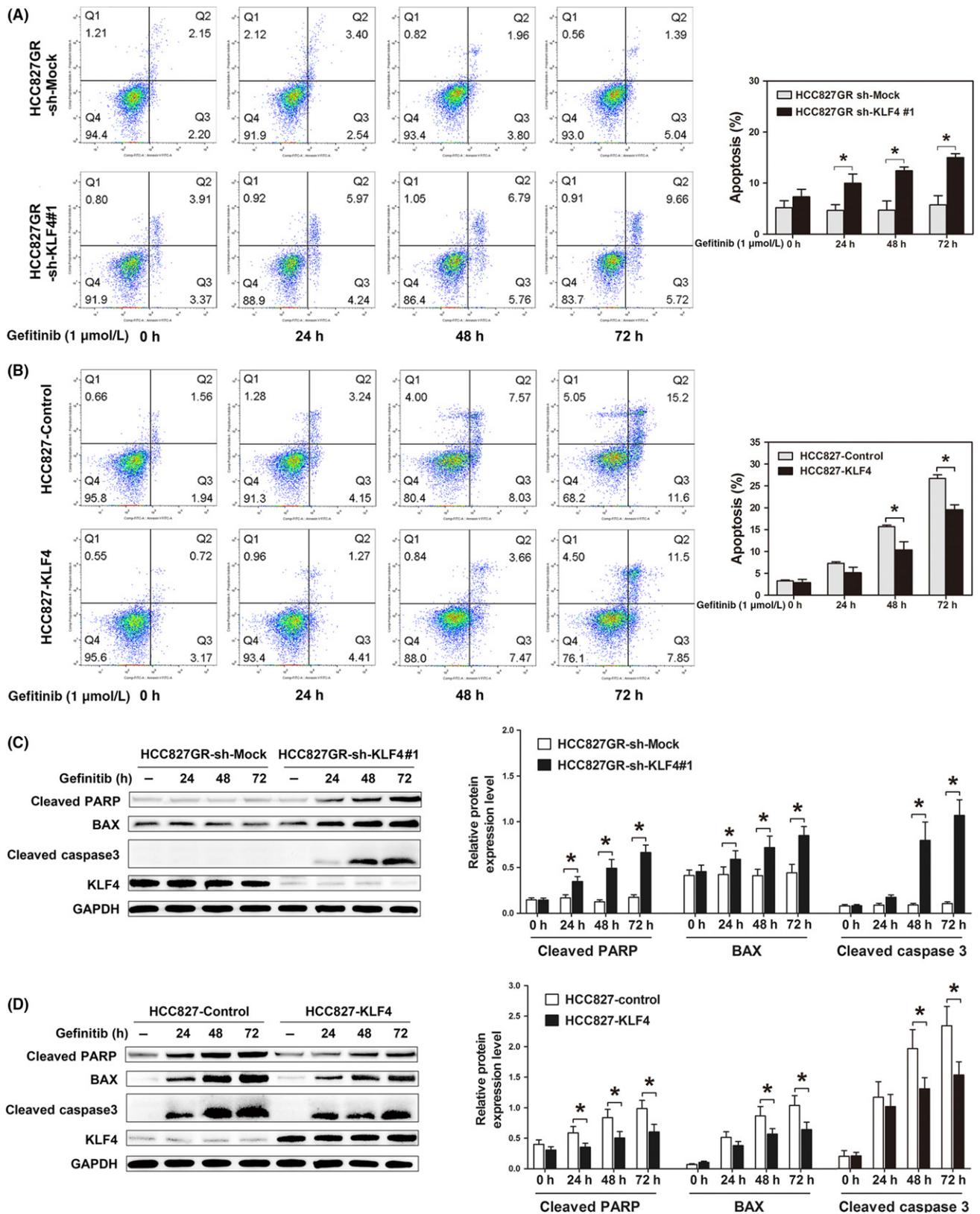


**FIGURE 3** Overexpression of Krüppel-like factor 4 (KLF4) reduces tumorigenic properties and gefitinib sensitivity in gefitinib-sensitive non-small-cell lung cancer cells. A,B, Clonogenic assays (left and middle panels) and MTS assays (right panels) were carried out to measure proliferation of KLF4-overexpressing and control groups of HCC4006 (A) and HCC827 (B) cells treated with 1 μmol/L gefitinib or vehicle (DMSO). Overexpression of KLF4 reduced the inhibition of gefitinib on the proliferation of gefitinib-sensitive HCC4006 and HCC827 cells. Statistical results are represented as mean ± SD (n = 3). C,D, Overexpression of KLF4 reduced tumorigenic properties and gefitinib sensitivity of HCC827 cells. Representative photographs of nude mice from each group inoculated with HCC827-Control or HCC827-KLF4 cells after 3 wk of treatment with gefitinib (50 mg/kg/d) or vehicle (0.5% Tween-80). Tumors dissected from each group are shown (C). Relative tumor volume curves of nude mice in each group are shown (D). Statistical results are represented as mean ± SD (n = 6). \*HCC827-Control group vs HCC827-KLF4 group,  $P < .05$ . \*\*Treatment with gefitinib (+G) vs vehicle in HCC827-KLF4 groups,  $P < .05$ . hTERT, human telomerase reverse transcriptase

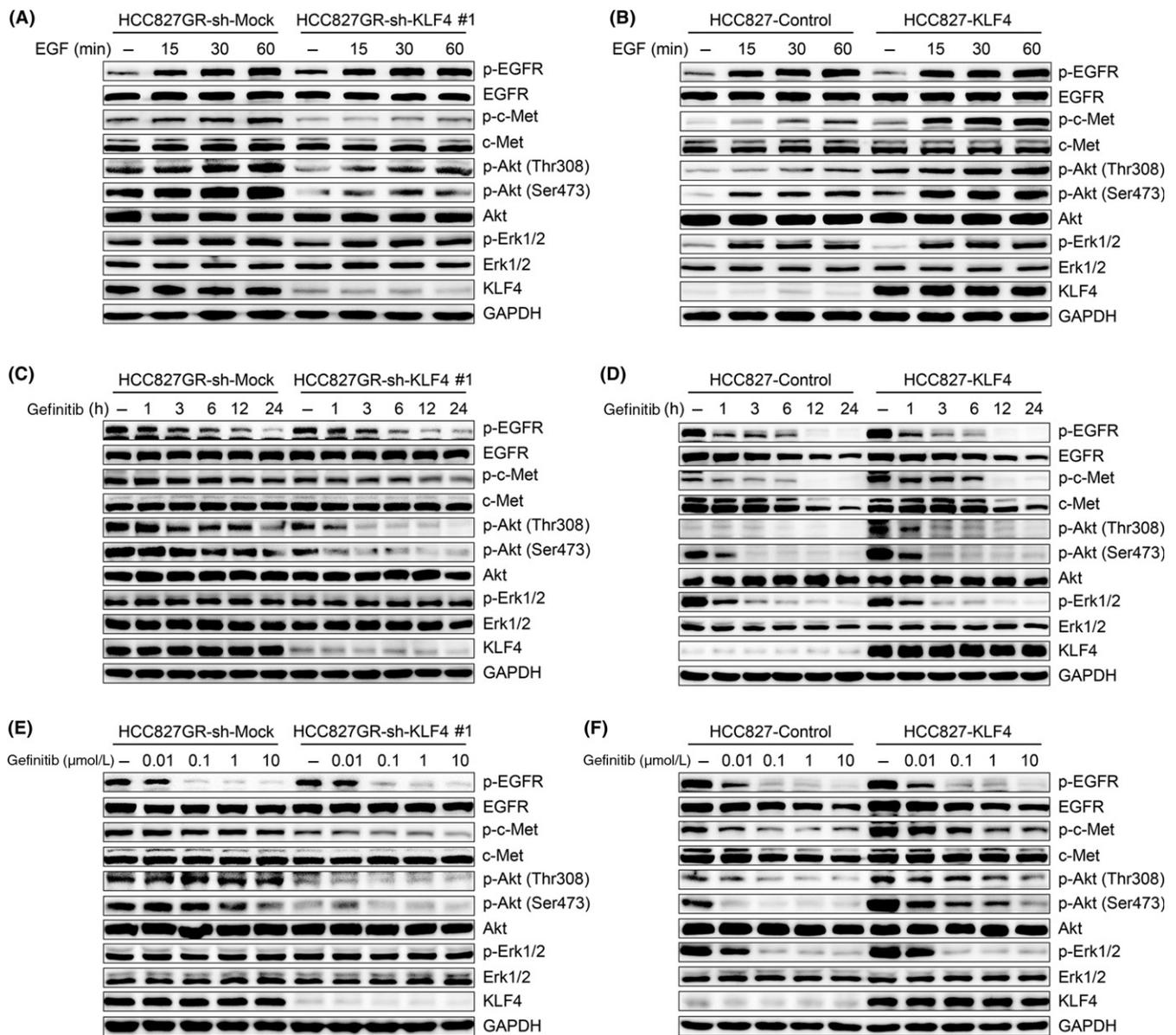
#### 4 | DISCUSSION

Uncontrolled activation of c-Met, such as *c-Met* and *HGF* amplification or mutation, has been reported as an important cause of acquired resistance of NSCLC to EGFR-TKI and is correlated with

poor clinical outcomes.<sup>33,34</sup> When binding to the receptor of c-Met, HGF can phosphorylate c-Met tyrosine kinase and subsequently activate the downstream ERBB3/PI3K/Akt signaling pathway, which promotes cell proliferation, migration, survival, and anti-apoptosis effects.<sup>34,35</sup> Moreover, c-Met overexpression is also detected in



**FIGURE 4** Krüppel-like factor 4 (KLF4) regulates gefitinib-induced cell apoptosis in HCC827GR and HCC827 cell lines. A, Flow cytometry analysis revealed that knockdown of KLF4 increased the apoptosis rate of HCC827GR cells. B, KLF4 overexpression reduced the apoptosis rate of HCC827GR cells after treatment with 1  $\mu\text{mol/L}$  gefitinib. C, Western blot analysis revealed that knockdown of KLF4 increased expression of the proteins cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase3, and BCL2-associated X (BAX) after treatment with 1  $\mu\text{mol/L}$  gefitinib. D, Opposite results of apoptosis-related protein expression were observed in HCC827 cells overexpressing KLF4. Statistical results are represented as mean  $\pm$  SD ( $n = 3$ ). \*Statistically significant difference ( $P < .05$ )

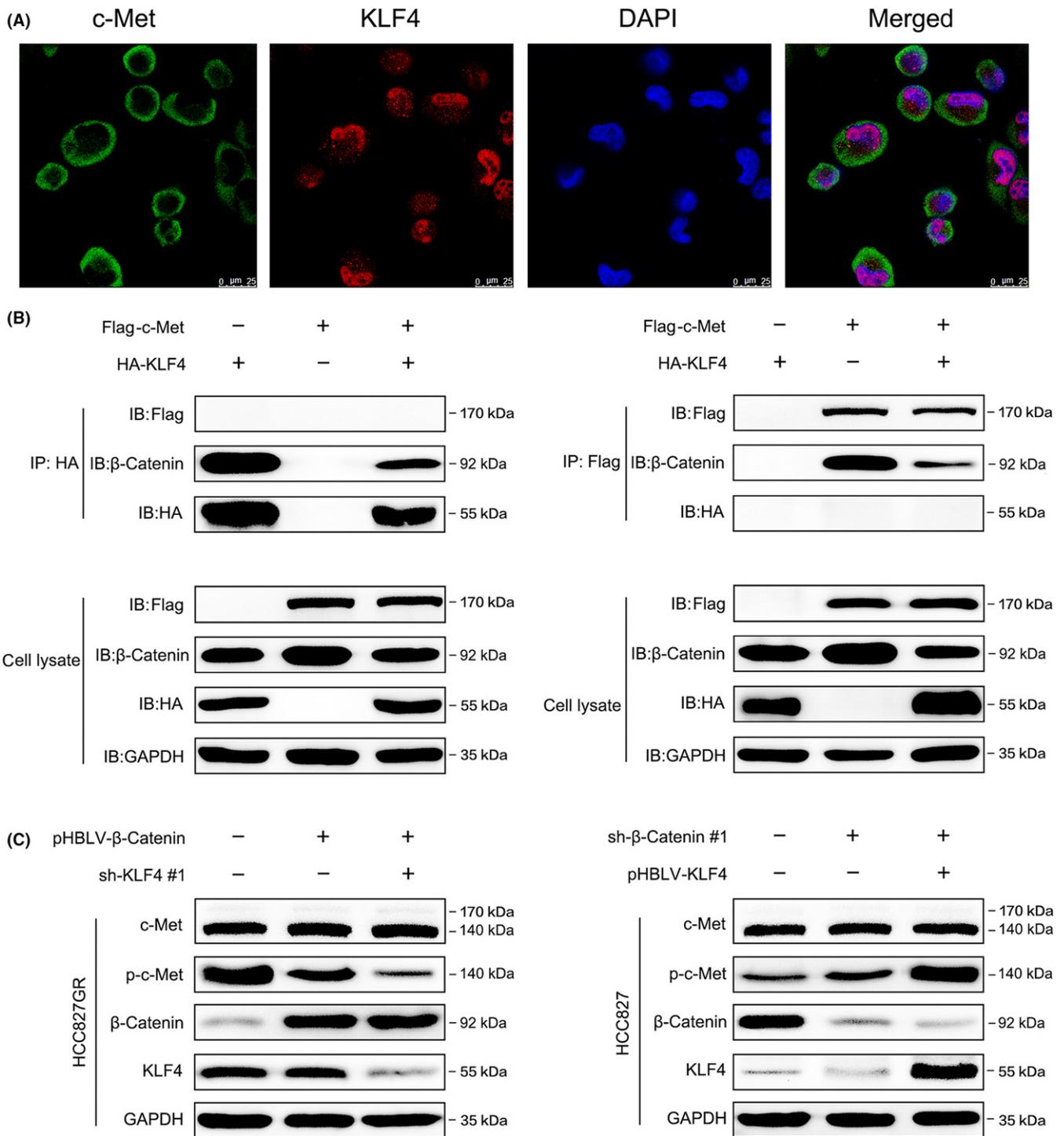


**FIGURE 5** Krüppel-like factor 4 (KLF4) regulates the c-Met/Akt signaling pathway. A,B, Stable KLF4-knockdown and KLF4-overexpressing cells were starved for 24 h and then treated with epidermal growth factor (EGF) (0 or 100 ng/mL) for various times. Western blot analysis revealed that EGF-induced phosphorylation (p-) of c-Met and Akt was repressed by knockdown of KLF4 (A) and promoted by overexpression of KLF4 (B), but phosphorylated epidermal growth factor receptor (p-EGFR), p-ERK1/2, total EGFR, c-Met, Akt, and Erk1/2 levels were not altered by KLF4 in either group (A,B) compared with levels in the control group. C-F, Stable KLF4-knockdown and KLF4-overexpressing cells were treated with various concentrations of gefitinib (6 h; C,D) or gefitinib (1  $\mu\text{mol/L}$ ; E,F) for various times. Western blot analysis revealed that gefitinib-induced inhibition of phosphorylation of c-Met and Akt was aggravated by knockdown of KLF4 with dose-dependent (C) and time-dependent (E) changes, but it was attenuated by overexpression of KLF4 with similar dose-dependent (D) and time-dependent (F) changes. Levels of p-EGFR, p-ERK1/2, total EGFR, c-Met, Akt, and Erk1/2 were not altered by KLF4 in either group (C-F)

NSCLC without EGFR-TKI treatment,<sup>7</sup> as our study showed (Figure 1C), which can result in primary resistance of NSCLC to EGFR-TKI.

Previous studies revealed that KLF4, a Krüppel-like transcription factor, has multiple roles in cellular processes, vascular functions, and inflammatory reactions.<sup>11</sup> Furthermore, as an ambiguous cancer-associated gene, KLF4 functions as a context-dependent oncogene or tumor suppressor gene in different cancer types.<sup>12,13</sup> In the present study, we observed that knockdown of KLF4 increases tumorigenic properties in NSCLC cells without c-Met overexpression

(Figure S1). Recent studies have shown that KLF4 directly binds to the promoter region of hTERT and suppresses hTERT expression.<sup>22,23</sup> We also observed that hTERT was upregulated by knockdown of KLF4 in NSCLC cells without c-Met overexpression (Figure S1). Therefore, the mechanism of KLF4, when functioning as a tumor-suppressor gene, might be a direct downregulation of the expression of hTERT in NSCLC cells without c-Met overexpression. However, we observed that KLF4 was dramatically upregulated in NSCLC cell lines and tumor tissues with c-Met overexpression



**FIGURE 6** Krüppel-like factor 4 (KLF4) represses the expression of  $\beta$ -catenin and inhibits binding between c-Met and  $\beta$ -catenin in non-small-cell lung cancer cells. A, Immunofluorescence double-labeling of c-Met and KLF4 in HCC827GR cells. c-Met is located in the cell membrane and KLF4 is located in the nucleus. B, Co-immunoprecipitation of c-Met,  $\beta$ -catenin, and KLF4 in A549 cells. Overexpression of *KLF4* downregulated the expression of  $\beta$ -catenin. Both KLF4 (left panels) and c-Met (right panels) interact with  $\beta$ -catenin, but KLF4 inhibited binding between c-Met and  $\beta$ -catenin (right panels). C, Overexpression of  $\beta$ -catenin inhibited phosphorylation of c-Met (left panels), whereas knockdown of  $\beta$ -catenin augmented its phosphorylation (right panels). IB, immunoblot; IP, immunoprecipitant

(Figure 1). Moreover, knockdown of KLF4 slightly increased hTERT expression (Figure 2), but it significantly inactivated c-Met and the downstream signaling pathway (Figure 5C,E), which resulted in the inhibition of proliferation and recovery of gefitinib sensitivity in c-

Met-amplified NSCLC cells (Figure 2). These results indicated that *KLF4* might function as an oncogene and contributes to the formation of gefitinib resistance in c-Met amplification-mediated gefitinib-resistant NSCLC cells. This tumorigenic discrepancy indicates that

*KLF4* can function as a tumor suppressor or an oncogene, depending on the *c-Met* status in NSCLC cells.<sup>29</sup>

Gefitinib can induce apoptosis by activating the caspase cascade in NSCLC cells carrying the sensitive mutation of *EGFR*.<sup>36</sup> Krüppel-like factor 4 also has an ambiguous apoptosis-induced effect in bladder cancer cells, breast cancer cells, and plasma cells.<sup>37-40</sup> The present study revealed that knockdown of *KLF4* promoted gefitinib-induced apoptosis by upregulating expression of apoptosis-related proteins in *c-Met*-overexpressing NSCLC cells (Figure 4A,C) but not in NSCLC cells without *c-Met* overexpression (Figure S2), whereas overexpression of *KLF4* suppressed gefitinib-induced apoptosis in gefitinib-sensitive cells (Figure 4B,D). This anti-apoptotic activity of *KLF4* could be explained by its ability to suppress *p53* expression by directly acting on the promoter<sup>41</sup> and inhibiting *p53* to transactivate expression of the pro-apoptotic gene *BAX*.<sup>42</sup>

One of the most important mechanisms of gefitinib resistance in NSCLC patients is the *c-Met* amplification, which leads to activation of the *EGFR* downstream PI3K/Akt signaling pathway.<sup>10</sup> However, the molecular mechanisms responsible for *c-Met* amplification in NSCLC patients after receiving gefitinib treatment have not been fully elucidated. Research showed that knockdown of *KLF4* inhibited the PI3K/Akt signaling pathway by regulating the promoter region of *p110 $\delta$*  in this pathway.<sup>43</sup> In the present study, we observed that knockdown of *KLF4* in HCC827GR cells inhibited *c-Met* and Akt activation, and overexpression of *KLF4* in HCC827 cells increased *c-Met* and Akt activation (Figure 5). These results suggest a mechanism by which *KLF4* contributed to gefitinib resistance in NSCLC cells with *c-Met* amplification by targeting the *c-Met*/Akt signaling pathway.

As a transcription factor, overexpression of *KLF4* downregulated the expression of  $\beta$ -catenin (Figure 6B), which is a direct target of *Klf4* transcriptional repression.<sup>32</sup> Furthermore, CoIP results showed that both *KLF4* and *c-Met* interacted with  $\beta$ -catenin, as reported in previous studies<sup>44,45</sup> and overexpression of *KLF4* effectively blocked binding between *c-Met* and  $\beta$ -catenin (Figure 6B, right panel), these results suggest that *KLF4* directly interferes with binding between *c-Met* and  $\beta$ -catenin. Further study showed that  $\beta$ -catenin inhibited phosphorylation of *c-Met* (Figure 6C), which suggest that  $\beta$ -catenin inhibits the activation of *c-Met* by directly interacting with *c-Met*.

Several prior studies showed that *c-Met* activation could increase *KLF4* expression in *in vitro* experiments.<sup>25,26,31</sup> In combination with our experimental results, we concluded that *c-Met* overexpression in NSCLC increased *KLF4* expression, which, in turn, activated the *c-Met*/Akt signaling pathway by repressing the expression of  $\beta$ -catenin and interfering with the inhibition of  $\beta$ -catenin on phosphorylation of *c-Met* and induced the formation of gefitinib resistance. However, several limitations still exist in our study. In future studies, we plan to explore more target genes or proteins of *c-Met* and *KLF4* in the process of gefitinib resistance and verify them in clinical samples.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ORCID

Wei Feng  <http://orcid.org/0000-0003-0065-6539>

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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