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

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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.1 Although the pathogenesis of lung cancer is complex, genetic susceptibility variants are a major cause.1,2 The most prominent genetic factor associated with the responsiveness to NSCLC treatment is a mutation in EGFR,3 which can activate the downstream PI3K/Akt/mTOR and KRas/Raf/Mek/Erk pathways to promote the malignant progression of NSCLC.4 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.4,5 However, many NSCLC patients will acquire resistance to gefitinib after a median of 9-14 months of treatment.6 The T790M of EGFR is the most common EGFR-TKI resistance mechanism, which confers resistance by increasing the ATP affinity.7 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.8,9 c-Met amplification 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 β-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

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INTRODUCTION

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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.10

Krüppel-like factor 4 (KLF4), as a transcription factor, is expressed in the epithelium of a variety of tissues11 and has multiple roles in physiological and disease processes, such as cellular processes, vascular functions, and tumorigenesis.11,12 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.12,13 KLF4 functions as a tumor suppressor gene to suppress the proliferation, invasion, and metastasis of tumor cells in kidney cancer,14 gastric cancer,15 and prostate cancer,16 but it was identified as an oncogene in breast cancer.17 Paradoxically, KLF4 functions as both an oncogene and tumor suppressor gene in colon cancer18,19 and skin cancer.20,21 Several studies have shown that tumor tissues had lower KLF4 levels compared with normal adjacent tissues.22-24 KLF4 might function as a tumor suppressor gene to suppress lung cancer growth by inhibiting human telomerase reverse transcriptase (hTERT) and MAPK signaling.22 KLF4 deletion can promote lung cancer formation and progression by activating the mutated oncogene KRAS.23 Another study revealed that activation of oncogenic c-Met could increase KLF4 expression in glioblastoma cells and glioblastoma stem cells.25,26

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 molecular 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°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.27 Data were analyzed by relative quantitation using the ΔΔCt method.27 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.28

2.4 | Lentiviral infection

Lentivirus packaging was carried out as previously described.29 Briefly, 293T cells were co-transfected with an appropriate proportion (4:3:1) of the lentivirus plasmids (plko.1-sh-KLF4, plko.1-sh-β-Catenin, pHBVL-Flag-c-Met, pHBVL-HA-KLF4, pHBVL-KLF4, and pHBVL-β-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 μg/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 × 10^3 cells/well in 200 μL) into 96-well plates and divided into the gefitinib group (four wells) and the control group (four wells). After incubation for an additional 0, 24, 48, or 72 hours, 20 μL 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 × 10^3 cells/well) in a 6-well plate and divided into the gefitinib and control groups and then treated with gefitinib (1 μmol/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 × 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 μmol/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 FACScverse (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 μg) were separated by 10% SDS-PAGE and transferred to 0.45-μm 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 Tyr1068, c-Met, p-c-Met Tyr1234/1235 Akt, p-Akt Ser473, p-Akt Thr308, Erk, p-Erk Thr202/Tyr204, cleaved poly(ADP-ribose) polymerase, cleaved caspase3 (Cell Signaling Technology, Boston, MA, USA), hTERT, hepatocyte growth factor (HGF), KLF4 (Abcam, Cambridge, USA), 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 × 10⁶ cells/100 μL) in the left axilla. When all tumors reached a mean volume of 50 mm³, 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. 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: 

\[ V = \frac{\text{length} \times \text{width}^2}{2} \]

The relative tumor volume (RTV) was calculated by the following formula: 

\[ \text{RTV} = \frac{V_{\text{tumor}}}{V_0} \]

where V₀ 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 ± 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: n = 3, P < .05). These results are consistent with previous studies, which reported that c-Met activation increased KLF4 expression in vitro experiments. In addition, no significant correlation between KLF4 and HGF was found in Figure 1B. 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.

| 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.
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 previously reported.22 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 μmol/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.
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 μmol/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 β-catenin and inhibits binding between c-Met and β-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 the expression of β-catenin and its binding to c-Met were not altered by KLF4 in NSCLC cells (Figure 6). However, knocking down KLF4 significantly decreased β-catenin expression (Figure 6A,B), and overexpression of KLF4 significantly inhibited the interaction between β-catenin and c-Met in HCC827 cells (Figures 6C,D; n = 3, P < .05). Furthermore, KLF4 did not change the phosphorylation of EGF, 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.

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

| Patient | Specimen   | Pathological type           | EGFR status | c-Met 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       | c-Met amplification |
| 8       | Tumor tissue | Adenocarcinoma              | L858R + T790M | WT                  |
| 9       | Tumor tissue | Adenocarcinoma              | WT          | c-Met 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          | WT                  |
| 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.
catenin (Ctnnb1) by targeting its promoter.\textsuperscript{32} Our results showed that overexpression of KLF4 downregulated the expression of β-catenin (Figure 6B). Furthermore, CoIP results showed that both KLF4 and c-Met interact with β-catenin, but KLF4 inhibited binding between c-Met and β-catenin (Figure 6B, right panel). Further study showed that overexpression of β-catenin inhibited phosphorylation of c-Met, whereas knockdown of β-catenin augmented its phosphorylation (Figure 6C). This dephosphorylation of c-Met by β-catenin could be explained by the interaction between β-catenin and c-Met, which inhibited phosphorylation of c-Met.

**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 μmol/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 ± 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 ± 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.
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. 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

poor clinical outcomes. 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. 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 μmol/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 μmol/L gefitinib. D, Opposite results of apoptosis-related protein expression were observed in HCC827 cells overexpressing KLF4. Statistical results are represented as mean ± SD (n = 3). *Statistically significant difference (P < .05).
NSCLC without EGFR-TKI treatment, as our study showed (Figure 1C), which can result in primary resistance of NSCLC to EGFR-TKI.

Previous studies revealed that KLF4, a Kruppel-like transcription factor, has multiple roles in cellular processes, vascular functions, and inflammatory reactions. Furthermore, as an ambiguous cancer-associated gene, KLF4 functions as a context-dependent oncogene or tumor suppressor gene in different cancer types. 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. We also observed that hTERT was upregulated 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).

**FIGURE 5** Kruppel-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 μmol/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).
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.29

Gefitinib can induce apoptosis by activating the caspase cascade in NSCLC cells carrying the sensitive mutation of EGFR.36 Krüppel-like factor 4 also has an ambiguous apoptosis-induced effect in bladder cancer cells, breast cancer cells, and plasma cells.37–40 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 and inhibiting p53 to transactivate expression of the pro-apoptotic gene BAX.41

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.10 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 p110α in this pathway.42 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 β-catenin (Figure 6B), which is a direct target of KLF4 transcriptional repression.32 Furthermore, CoIP results showed that both KLF4 and c-Met interacted with β-catenin, as reported in previous studies and overexpression of KLF4 effectively blocked binding between c-Met and β-catenin (Figure 6B, right panel), these results suggest that KLF4 directly interferes with binding between c-Met and β-catenin. Further study showed that β-catenin inhibited phosphorylation of c-Met (Figure 6C), which suggest that β-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.25,26,31 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 β-catenin and interfering with the inhibition of β-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.

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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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