

Hypoxia-inducible factor-1 signaling pathway influences the sensitivity of HCC827 cells to gefitinib

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Abstract. The majority of patients with non-small cell lung cancer (NSCLC) with activating epidermal growth factor receptor (EGFR) mutations inevitably progress in stage despite an initial substantial and rapid response to EGFR-tyrosine kinase inhibitors (EGFR-TKIs). Previous research indicates that hypoxia may be associated with resistance to EGFR-TKIs in EGFR mutation-positive NSCLC. Therefore, the present study regulated the activity of hypoxia-inducible factor-1 (HIF-1) signaling pathway to observe if it is able to alter the sensitivity of lung cancer cells to gefitinib. The present study selected 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) and dimethyloxalylglycine (DMOG) as a HIF-1 signaling pathway inhibitor and activator, respectively, on HCC827 cells. Cells were incubated with different treatments for different durations: A blank control, DMOG, gefitinib, or DMOG and gefitinib combined, for 36 and 48 h; and then a blank control, YC-1, gefitinib, or YC-1 and gefitinib combined, for 16 and 28 h. A western blot analysis assay was performed to evaluate the protein expression levels of HIF-1 α and phosphorylated hepatocyte growth factor receptor (p-MET), an MTT assay was used to determine cell proliferation, a colony formation assay was used to investigate the colony-forming ability and a wound healing assay was used to test the cell migration ability. Additionally, Pearson's correlation

analysis was used to evaluate the correlation between p-Met and HIF-1 α expression levels. Finally, it was identified that gefitinib and DMOG combined notably improve the growth and cell migration ability of HCC827 cells, compared with gefitinib alone. When gefitinib and YC-1 were combined, the inhibiting effect on the growth and cell migration ability of HCC827 cells was substantially enhanced, compared with the control cells. Pearson's correlation analysis revealed that the p-Met expression level had a strong positive correlation with HIF-1 α expression levels. Thus, it was concluded that the HIF-1 signaling pathway influences the sensitivity of HCC827 cells to gefitinib. The positive correlation between p-Met and HIF-1 α expression levels may be the underlying mechanism of the HIF-1 signaling pathway influencing the sensitivity of HCC827 cells to gefitinib.

Introduction

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-associated mortality globally (1). The acquired resistance of anticancer drugs remains a key obstacle for improving the prognosis of patients with NSCLC (2). Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) have been selected clinically as the first-line treatment for patients with NSCLC by activating EGFR mutations (3-5). However, the disease stage of the majority of patients inevitably progresses despite an initial substantial and rapid response to EGFR-TKIs (6). Previous studies indicated that human EGFR-2 amplification, original or induced T790M mutation, activated secondary signaling, including phosphatidylinositol 3-kinase mutation or hepatocyte growth factor receptor (MET) proto-oncogene, and receptor tyrosine kinase amplification may result in acquired EGFR-TKIs resistance (6-8). However, the initial mechanism for the acquired resistance of EGFR-TKIs remains unclear.

Hypoxia is a notable feature of solid tumor types, including lung cancer (9). Compared with tumors under oxygen-rich conditions, hypoxic tumors are more resistant to radiation and chemotherapy, more invasive, genetically unstable, resist apoptosis and have increased metastatic potential (10). Hypoxia activates the hypoxia-inducible factor-1 (HIF-1) signaling pathway, which mediates the primary biological effects of hypoxia (9). HIF-1 consists of an α and β subunit, and HIF-1 α

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Abbreviations: NSCLC, non-small cell lung cancer; EGFR-TKIs, epidermal growth factor receptor-tyrosine kinase inhibitors; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; HIF-1, hypoxia-inducible factor-1; DMOG, dimethyloxalylglycine

Key words: hypoxia-inducible factor-1, gefitinib, oxalylglycine, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole, phosphorylated hepatocyte growth factor receptor

is the functional part (11). Previous research indicates that hypoxia increases the population of lung cancer stem cells resistant to gefitinib in EGFR mutation-positive NSCLC, and the HIF-1 signaling pathway is activated in EGFR-TKI-resistant lung cancer cells (12,13). Thus, the HIF-1 signaling pathway was targeted as a potential factor to influence the sensitivity of lung cancer cells to EGFR-TKIs.

In the present study, the activity of the HIF-1 signaling pathway was regulated to observe if it was able to alter change the sensitivity of lung cancer cells to EGFR-TKIs. The present study selected 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) and dimethyloxallylglycine (DMOG) as a HIF-1 signaling pathway inhibitor and activator, respectively. YC-1 is a chemically synthetic benzyl indazole (14). It had been revealed to be able to downregulate HIF-1 α expression and was indicated as a novel HIF-1 α inhibitor (15). The prolyl hydroxylase inhibitor DMOG has been used as an activator of the HIF-1 signaling pathway (16). It physiologically simulates a low oxygen environment by blocking the degradation of HIF, and inducing chemical hypoxia (16,17).

Gefitinib was selected as the representative EGFR-TKI. HCC827, the gefitinib hypersensitive EGFR exon 19 mutant NSCLC cell line (8), was selected for the present study. Previous research demonstrated that MET amplification is the mechanism of acquired resistance against gefitinib in HCC827-GR, the gefitinib resistant cell line generated by exposing HCC827 cells to increasing concentrations of gefitinib (8,18). Additionally, previous research indicated that HIF-1 α is involved in the regulation of MET levels by EGFR, and EGFR regulation of MET levels in EGFR-TKIs sensitive cell lines occurs through the HIF-1 signaling pathway in a hypoxia-independent manner (19). Nevertheless, in an EGFR-TKI-resistant cell line with MET amplification, this regulation was lost (8). Subsequently, for the present study it was speculated that the HIF-1 signaling pathway, but not the EGFR pathway, may regulate MET levels in EGFR-TKI-resistant cell lines caused by MET amplification. HCC827 cells can become an EGFR-TKI-resistant cell line following MET amplification (18); therefore, the HCC827 cell line was selected for the present study. In addition to examining the influence of the HIF-1 signaling pathway on the sensitivity of HCC827 cells to gefitinib, the present study also aimed to investigate the associated mechanisms of this resistance and to detect the levels of MET, in order to clarify whether MET levels are associated with the sensitivity change of HCC827 cells to gefitinib caused by the HIF-1 signaling pathway.

Materials and methods

Reagents. Reagents and suppliers were as follows: Gefitinib (AstraZeneca UK Limited, Macclesfield, UK); YC-1; DMOG; MTT cell viability kit (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); penicillin-streptomycin solution (Beyotime Institute of Biotechnology, Shanghai, China); antibodies p-Met and HIF-1 α (cat. no. ab5662 and ab82832, respectively; Abcam, Cambridge, MA, USA); and RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell line and cell culture. The present study was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province (Hangzhou, China). Human commercially available HCC827 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured under standard conditions (37°C and 5% CO₂) and in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin solution (standard medium). HCC827 cells were observed regularly under a light microscope (x40; BX-42; Olympus Corporation, Tokyo, Japan).

Cells were separated into four groups to research the effect of HIF-1 signaling pathway upregulation on the sensitivity of HCC827 cells to gefitinib: A blank control group; a DMOG group; a gefitinib group; and a DMOG and gefitinib combined group. Every group contained two subgroups that were treated for different durations. For the blank control group, HCC827 cells were cultured for 36 and 48 h (37°C and 5% CO₂) in standard medium, as aforementioned. For the DMOG group, HCC827 cells were cultured for 36 and 48 h (37°C and 5% CO₂) in standard medium with 2 mM DMOG. For the gefitinib group, after culturing HCC827 cells in standard medium for 24 h, gefitinib was added to the medium at a final concentration of 20 nM and treated for 36 and 48 h. For the DMOG and gefitinib combined group, following the culture of HCC827 cells in standard medium with 2 mM DMOG for 24 h, gefitinib was added to the existing medium at a final concentration of 20 nM and treated for 36 and 48 h.

Similarly, cells were also separated into four groups to examine the effect of HIF-1 signaling pathway downregulation on the sensitivity of HCC827 cells to gefitinib: A blank control group, a YC-1 group, a gefitinib group, and a YC-1 and gefitinib group. For the blank control group, the HCC827 cells were cultured for 16 and 28 h (37°C and 5% CO₂) in standard medium. For the YC-1 group, HCC827 cells were cultured for 16 and 28 h (37°C and 5% CO₂) in standard medium with 40 μ M YC-1. For the gefitinib group, following the culturing of HCC827 cells in standard medium for 4 h, gefitinib was added to the medium at a final concentration of 20 nM and treated for 16 and 28 h. For the YC-1 and gefitinib combined group, following the culture of HCC827 cells in standard medium with 40 μ M YC-1 for 4 h, gefitinib was added to the existing medium at a final concentration of 20 nM and treated for 16 and 28 h.

Western blot assay. Cells treated with the aforementioned different treatments were washed with PBS (0.01 M, pH 7.2-7.3 at 4°C) three times, and then treated with a lysis buffer containing 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1% Triton X-100 and inhibitors of protease and phosphates (Beyotime Institute of Biotechnology) on ice for 30 min. The cell lysis products were centrifuged for 15 min at 12,000 \times g in a 4°C refrigerated centrifuge and the supernatants were collected. The final protein concentration was measured using a Bicinchoninic Acid protein kit, according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.), and supernatants were boiled for 5 min at 100°C. Subsequently, 100 μ g protein lysates were separated using 12% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes and the membranes were blocked with 5% skimmed milk with

TBS and 20% Tween-20 (TBST) at room temperature for 2 h. A total of 137 mM NaCl, 20 mM Tris and 0.05% Tween-20 were contained in TBST of which the pH was adjusted with HCl to pH 7.5. The blotted membrane was incubated with primary antibodies against p-Met (1:500), HIF-1 α (1:500) and GAPDH (1:1,000; ab9484; Abcam) at room temperature for 2 h. The membrane was washed with TBST three times and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. RABHRP1-10UL; 1:1,000; Sigma-Aldrich; Merck KGaA) at room temperature for 1.5 h. The immunoreactive bands were washed with TBST four times and observed using enhanced chemiluminescence plus detection reagent (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control. The densitometry of the bands was quantified with an UVP Gel Imaging System Labworks 4.6 software (UVP, LLC, Phoenix, AZ, USA).

MTT assay. HCC827 cells were seeded at a density of 2×10^4 cells/well in 96-well plates and maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Following overnight incubation (37°C and 5% CO₂), cells were exposed to different treatments (blank control, DMOG, gefitinib, and DMOG and gefitinib combined for 36 and 48 h; blank control, YC-1, gefitinib, and YC-1 and gefitinib combined for 16 and 28 h). Following treatments, an MTT reagent (Sigma-Aldrich; Merck KGaA) was added and cells were incubated at 37°C for 4 h. Subsequently, the medium was removed and 150 μ l dimethyl sulphoxide was added to dissolve the purple formazan salt crystals. Following this, the optical density was measured on a microplate reader at a wavelength of 490 nm.

Colony formation assay. Cells with different treatments as aforementioned were seeded onto culture plates. Cells were seeded at low density (300 cells/plate) and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin for 2-3 weeks, in a humidified atmosphere with 5% CO₂ at 37°C. Subsequently, the colonies were stained with 0.05% crystal violet solution for 20 min at room temperature. Finally, the number of colonies with >10 cells were counted under an inverted light microscope (x10; CKX41; Olympus Corporation).

Cell migration assay. Subsequent to the aforementioned treatments, HCC827 cells were plated into 6-well plates and cultured under serum starvation conditions, in RPMI-1640 medium without FBS, to a maximum of 60% confluence. A scratch was produced 16 h after serum starvation of the cells. Each well was wounded by scratching with a 10 μ l pipette tip, which was followed by PBS washes three times to remove cell debris. The gap distance of the wound was measured at three different sites using ImageJ Software (v. 1.48q; National Institutes of Health, Bethesda, MD, USA) in pixels. Wound closure was observed at 0, 24 and 48 h after wound simulation. Graphs were plotted against the percentage of the migration distance that the cells moved.

Statistical analysis. Statistical analyses were performed using SPSS software (version 16.0.0; SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean \pm standard deviation,

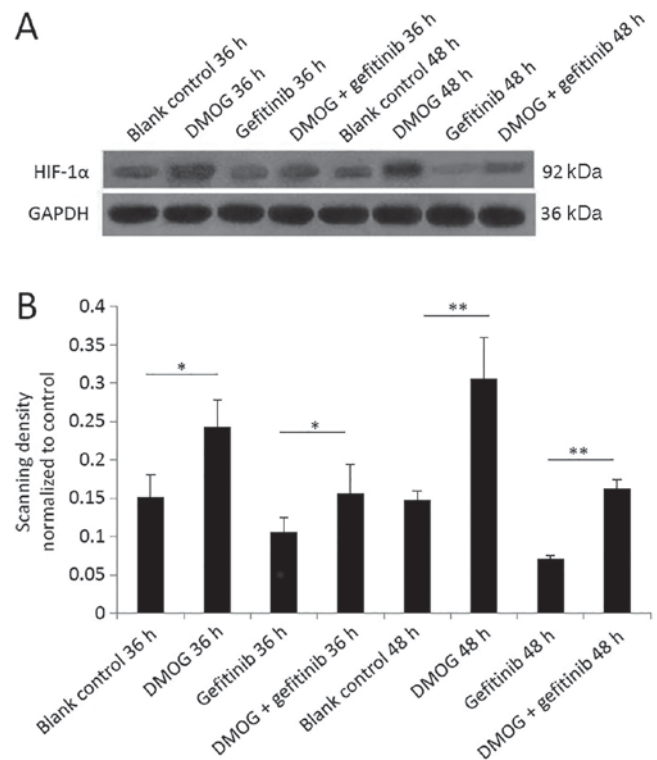


Figure 1. Expression of HIF-1 α in HCC827 cells with different treatments of DMOG and gefitinib. (A) Western blot analysis was used to examine HIF-1 α level in HCC827 cells with different treatments (blank control, DMOG, gefitinib, and DMOG and gefitinib combined for 36 and 48 h). Concentrations of DMOG and gefitinib used were 2 mM and 20 nM, respectively. (B) Bar graphs derived from densitometric scanning of the blots. Error bars represent the mean \pm standard deviation from three independent experiments. *P<0.05 and **P<0.01. HIF-1 α , hypoxia-inducible factor-1; DMOG, dimethylxalylglycine.

which was based on a minimum of three independent experiments. Differences between groups were compared using a two-way analysis of variance followed by a post hoc Tukey's test. Correlation between HIF-1 α levels and p-Met levels of HCC827 cells with different treatments was evaluated by Pearson's correlation. P<0.05 was considered to indicate a statistically significant difference.

Results

HIF-1 signaling pathway upregulation reduces the sensitivity of HCC827 cells to gefitinib. A western blot assay was performed to detect the expression of HIF-1 α in HCC827 cells with different treatments (blank control, DMOG, gefitinib, and DMOG and gefitinib combined for 36 and 48 h). As depicted in Fig. 1, the protein expression levels of HIF-1 α were significantly elevated in cells treated with DMOG, compared with the blank control for 36 and 48 h (P<0.05 and P<0.01, respectively). Similarly, levels of HIF-1 α were significantly elevated in cells treated with DMOG and gefitinib combined, compared with the gefitinib treatment, at 36 and 48 h (P<0.05 and P<0.01, respectively). This indicates the activation of the HIF-1 signaling pathway. The effect of HIF-1 signaling pathway upregulation on HCC827 cell proliferation was assessed by performing an MTT assay. When HCC827 cells were treated with DMOG and gefitinib combined, a significant increase in cell proliferation was observed, compared with

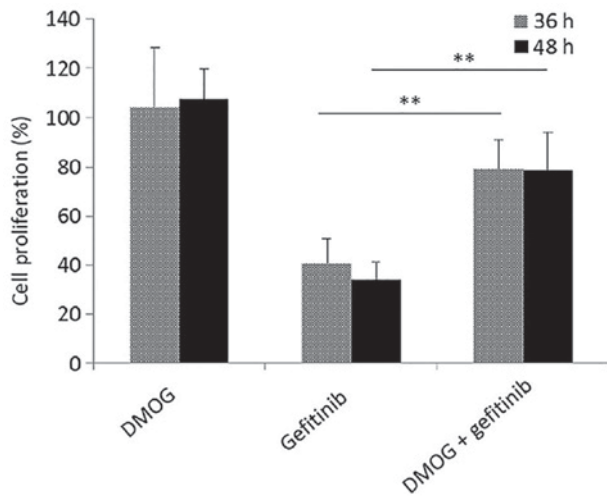


Figure 2. An MTT assay in HCC827 cells with different treatments of DMOG and gefitinib. An MTT assay was performed to determine the cell proliferation of HCC827 cells with different treatments (DMOG, gefitinib, and DMOG and gefitinib combined for 36 and 48 h). Concentrations of DMOG and gefitinib used were 2 mM and 20 nM, respectively. Error bars represent the mean \pm standard deviation from three independent experiments. ** $P < 0.01$. DMOG, dimethylxalylglycine.

gefitinib treated HCC827 cells ($P < 0.01$; Fig. 2). This indicates that HCC827 cells were less sensitive to gefitinib once DMOG was added, compared with gefitinib alone. Consistent with the results from the MTT assay, the gefitinib and DMOG combined treatment significantly improved the colony-forming ability of HCC827 cells, compared with gefitinib treatment alone, at 36 and 48 h ($P < 0.01$ and $P < 0.05$, respectively; Fig. 3). Additionally, it was revealed that DMOG treatment alone for 48 h significantly improved the colony-forming ability of HCC827 cells, compared with the blank control ($P < 0.05$; Fig. 3). In addition to the growth ability of HCC827 cells, the influence of HIF-1 signaling pathway upregulation on cell migration was examined. In a wound healing assay, treatment of gefitinib and DMOG combined for 36 and 48 h significantly enhanced cell migration, compared with gefitinib treatment alone ($P < 0.01$; Fig. 4), while treatment of DMOG alone for 24 h significantly enhanced the cell migration ability of HCC827 cells, compared with the blank control ($P < 0.05$; Fig. 4).

HIF-1 signaling pathway downregulation enhances the sensitivity of HCC827 cells to gefitinib. To demonstrate the effect of the downregulation of the HIF-1 signaling pathway, western blot analysis was performed to detect the expression of HIF-1 α in HCC827 cells with different treatments (blank control, YC-1, gefitinib, and YC-1 and gefitinib combined for 16 and 28 h). As depicted in Fig. 5, the protein expression levels of HIF-1 α were significantly reduced in cells treated with YC-1, compared with the blank control for 36 and 48 h ($P < 0.01$). Similarly, the levels of HIF-1 α were significantly reduced in cells treated with YC-1 and gefitinib combined, compared with the gefitinib treatment alone, for both 36 and 48 h ($P < 0.01$). To determine the sensitivity change of HCC827 cells to gefitinib subsequent to the downregulation of the HIF-1 signaling pathway, an MTT assay, a colony formation analysis and a wound-healing assay were performed. In the MTT assay, when HCC827 cells were treated with YC-1 and

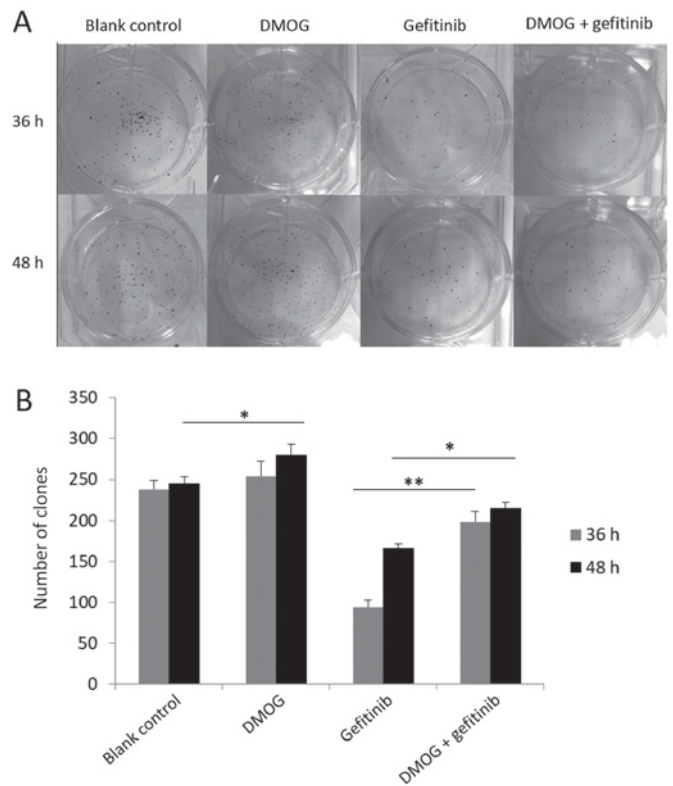


Figure 3. Colony formation analysis of HCC827 cells with different treatments of DMOG and gefitinib. (A) Colony formation analysis was performed to evaluate the colony-forming ability of HCC827 cells with different treatments (blank control, DMOG, gefitinib, and DMOG and gefitinib combined for 36 and 48 h). Concentrations of DMOG and gefitinib used were 2 mM and 20 nM, respectively. (B) Quantified results from three independent experiments. Error bars represent the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$. DMOG, dimethylxalylglycine.

gefitinib combined, a significant reduction in cell proliferation was observed, compared with gefitinib alone treated HCC827 cells ($P < 0.05$; Fig. 6). This indicated that the HCC827 cells became more sensitive to gefitinib following YC-1 treatment, whilst the HIF-1 signaling pathway was downregulated. YC-1 alone had a significant inhibitory effect on the colony-forming ability of HCC827 cells, compared with the blank control ($P < 0.05$; Fig. 7). Gefitinib and YC-1 combined treatment resulted in significant inhibitory effect on the colony-forming ability of HCC827 cells, compared with gefitinib treatment alone ($P < 0.01$; Fig. 7). In the wound healing assay, treatment with gefitinib and YC-1 combined significantly inhibited cell migration, compared with gefitinib treatment alone ($P < 0.01$; Fig. 8).

Association between p-Met level of HCC827 cell and the activity of HIF-1 signal pathway. Using western blot analysis, the protein expression levels of HIF-1 α and p-Met were examined. The protein expression levels of HIF-1 α in HCC827 cells with different treatments are depicted and described in Figs. 1 and 5. When HCC827 cells were treated with DMOG, the protein expression levels of HIF-1 α and p-Met were elevated, compared with the control cells ($P < 0.05$ and $P < 0.01$ for 36 and 48 h, respectively; Fig. 9). When HCC827 cells were treated with YC-1, the protein expression levels of HIF-1 α and p-Met were significantly reduced, compared with the blank control

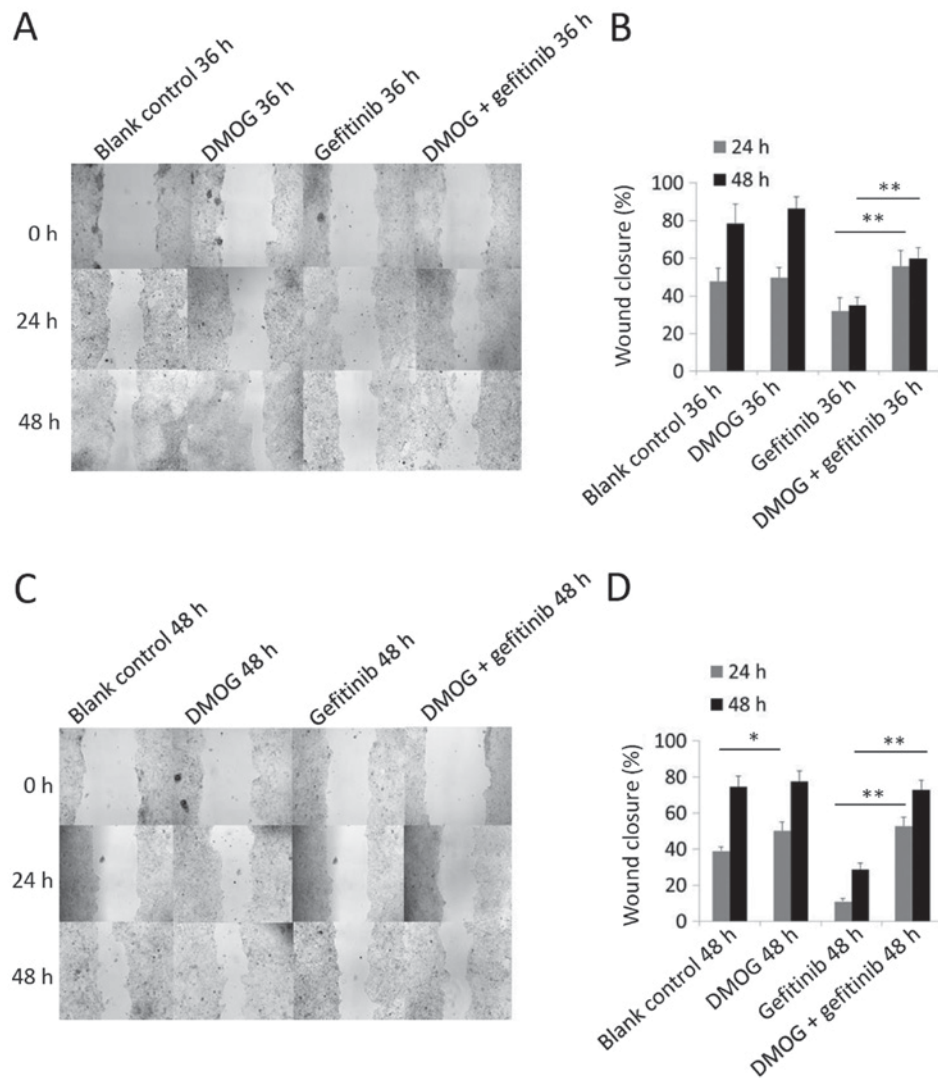


Figure 4. Wound healing assay of HCC827 cells with different treatments of DMOG and gefitinib. (A) A wound healing assay was performed to determine the cell migration ability of HCC827 cells with different treatments (blank control, DMOG, gefitinib, and DMOG and gefitinib combined for 36 h). Representative images captured 24 and 48 h after wounding (magnification, x100) depicting the cell migration. Concentrations of DMOG and gefitinib used were 2 mM and 20 nM, respectively. (B) Quantified wound-healing percentage of HCC827 cells 24 and 48 h after being wounded. Error bars represent the mean \pm standard deviation from three independent experiments. (C) A wound healing assay was performed to evaluate the cell migration ability of HCC827 cells with different treatments (blank control, DMOG, gefitinib, and DMOG and gefitinib combined for 48 h). Representative images photographed 24 and 48 h after wounding (magnification, x100) depicting the cell migration. Concentrations of DMOG and gefitinib used were 2 mM and 20 nM, respectively. (D) Quantified wound-healing percentage of HCC827 cells 24 and 48 h after being wounded. Error bars represent the mean \pm standard deviation from three independent experiments. * $P<0.05$ and ** $P<0.01$. DMOG, dimethylxalylglycine.

($P<0.01$ for 16 and 28 h; Fig. 9). It was revealed that the p-Met protein expression levels in HCC827 cell were associated with the protein expression levels of HIF-1 α . Based on these results, the correlation between p-Met and HIF-1 α protein levels in HCC827 cells was investigated by Pearson's correlation analysis (Fig. 10). The expression level of p-Met was significantly positively correlated with HIF-1 α levels ($r^2=0.978$, $P<0.01$).

Discussion

The present study revealed that the HIF-1 signaling pathway influenced the sensitivity of HCC827 cells to gefitinib, thus providing insights into the effect of the HIF-1 signaling pathway on acquired gefitinib resistance. Furthermore, p-Met levels exerted a strong positive correlation with HIF-1 α level, which may be the molecular mechanism underlying the

influence of HIF-1 signaling pathway on the sensitivity of HCC827 cells to gefitinib.

HIF-1 mediates the primary biological effects of hypoxia in tumorigenesis (20). Activation of HIF-1 transcription results in the upregulation of a number of genes, including vascular endothelial growth factor, insulin-like growth factor 2, telomerase reverse transcriptase, stroma-derived factor 1 and multidrug resistance 1, which encode proteins participating in tumor angiogenesis, cell proliferation, survival, invasion and therapy resistance (21,22). Accumulation of HIF-1 limits the effectiveness of radiotherapy and numerous cytotoxic drugs (23). For HIF-1 signaling in EGFR-TKI therapy resistance, previous research indicated that they may be associated. In an acquired EGFR-TKI resistant cell line, the upregulation of HIF-1 α was observed, compared with an EGFR-TKI sensitive cell line (13). Furthermore, hypoxia increased the

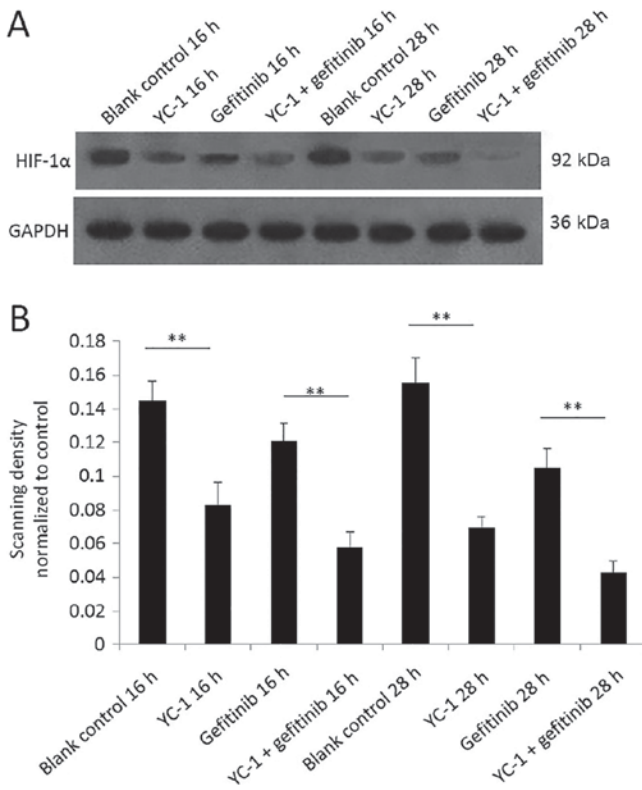


Figure 5. Expression of HIF-1 α in HCC827 cells with different treatments of YC-1 and gefitinib. (A) Western blot analysis was performed to assess HIF-1 α protein expression levels in HCC827 cells with different treatments (blank control, YC-1, gefitinib, and YC-1 and gefitinib combined for 16 and 28 h). Concentrations of YC-1 and gefitinib used were 40 μ M and 20 nM, respectively. (B) Quantified densitometric scanning of the blots. Error bars represent the mean \pm standard deviation from three independent experiments. ** $P < 0.01$. HIF-1 α , hypoxia-inducible factor-1; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

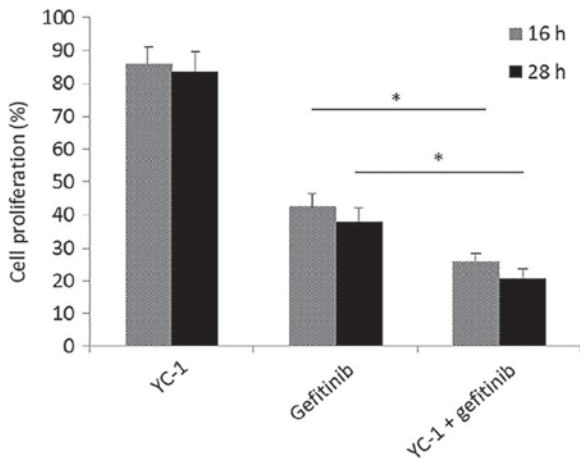


Figure 6. MTT assay in HCC827 cells with different treatments of YC-1 and gefitinib. An MTT assay was performed to assess the cell proliferation of HCC827 cells with different treatments (YC-1, gefitinib, and YC-1 and gefitinib combined for 16 and 28 h). Concentrations of YC-1 and gefitinib used were 40 μ M and 20 nM, respectively. Error bars represent the mean \pm standard deviation from three independent experiments. * $P < 0.05$. YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

population of lung cancer stem cells resistant to gefitinib in EGFR mutation-positive NSCLC (PC9 and HCC827 cells)

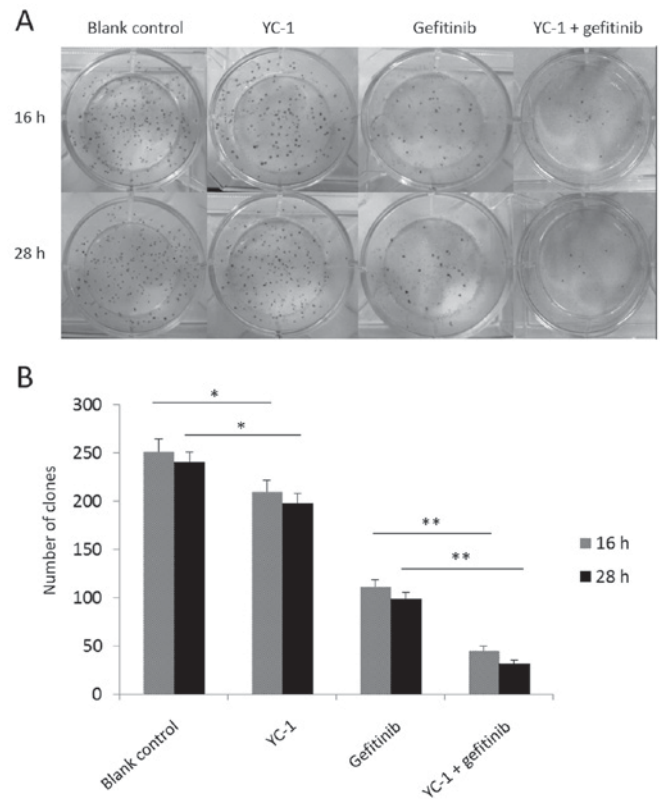


Figure 7. Colony formation analysis of HCC827 cells with different treatments of YC-1 and gefitinib. (A) A colony formation analysis was performed to determine the colony-forming ability of HCC827 cells with different treatments (blank control, YC-1, gefitinib, and YC-1 and gefitinib combined for 16 and 28 h). Concentrations of YC-1 and gefitinib used were 40 μ M and 20 nM, respectively. (B) Quantified results from three independent experiments. Error bars represent the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$. YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

by activating insulin-like growth factor 1 receptor (12). In the present study, it was observed that HIF-1 signaling pathway upregulation caused by DMOG is able to reduce the sensitivity of HCC827 cells to gefitinib (Figs. 2-4). This is in accordant with previous studies (24-26). This indicates that the HIF-1 signaling pathway may participate in the forming of acquired EGFR-TKIs resistance.

When the HIF-1 pathway is upregulated, vascular endothelial growth factor, epithelial-mesenchymal transition and membrane-type 4 matrix metalloproteinase are activated (27-29). In the present study, DMOG treatment alone for 48 h was able to promote the colony-forming and migrating ability of HCC827 cells (Figs. 3 and 4). DMOG achieves its effects on HCC827 cells through the upregulation of the HIF-1 pathway (30,31). When DMOG and gefitinib treatments were combined, the effect of gefitinib was substantially inhibited (Figs. 2-4). This may verify the importance of the HIF-1 pathway inhibition in NSCLC treatment.

YC-1, as an HIF-1 inhibitor, possesses antitumor effects itself (32). YC-1 can inhibit proliferation of breast cancer cells (33,34), however its effect on lung cancer is confined to its influence on enhancing radiotherapy sensitivity (35,36). The present study observed that the downregulation of the HIF-1 signaling pathway caused by YC-1 is able to enhance the sensitivity of HCC827 cells to gefitinib. When YC-1

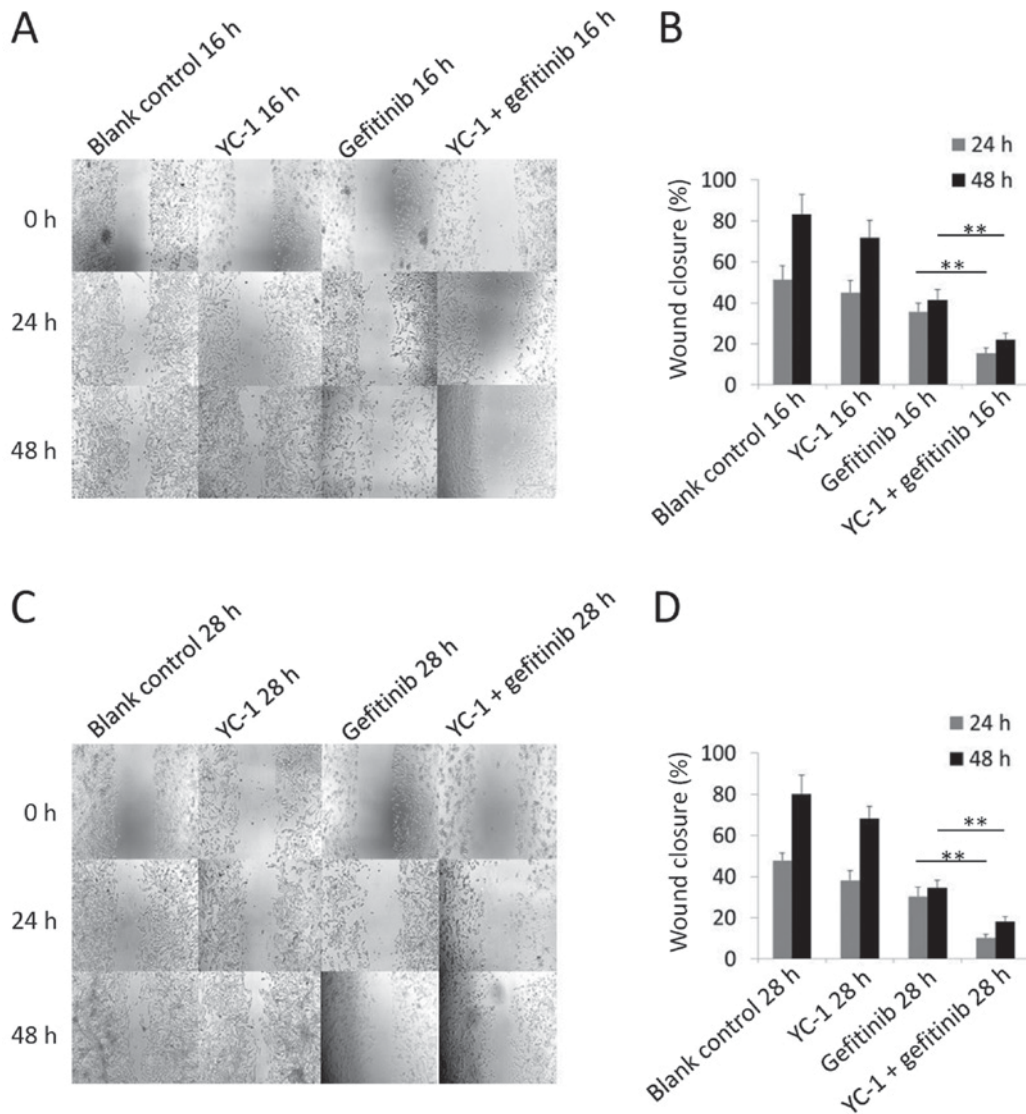


Figure 8. Wound healing assay of HCC827 cells with different treatments of YC-1 and gefitinib. (A) A wound healing assay was performed to determine the cell migration ability of HCC827 cells with different treatments (blank control, YC-1, gefitinib, and YC-1 and gefitinib combined for 16 h). Representative images captured 24 and 48 h after wounding (magnification, x100) are depicted. Concentrations of YC-1 and gefitinib used were 40 μ M and 20 nM, respectively. (B) Quantified wound-healing percentage of HCC827 cells 24 and 48 h after being wounded. Error bars represent the mean \pm standard deviation from three independent experiments. (C) A wound healing assay was performed to evaluate the cell migration ability of HCC827 cells with different treatments (blank control, YC-1, gefitinib, and YC-1 and gefitinib combined for 28 h). Representative images were captured 24 and 48 h after wounding (magnification, x100) are depicted. Concentrations of YC-1 and gefitinib used were 40 μ M and 20 nM, respectively. (D) Quantified wound-healing percentage of HCC827 cells 24 and 48 h after being wounded. Error bars represent the mean \pm standard deviation from three independent experiments. * P <0.05 and ** P <0.01. YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

was combined with gefitinib, the inhibiting effect on the colony-forming, cell migration and cell proliferation abilities of HCC827 cells was substantially enhanced, compared with gefitinib alone (Figs. 6-8). Due to the synergistic effect of YC-1 and gefitinib on HCC827 cells, it was speculated that YC-1 may enhance the sensitivity of acquired EGFR-TKI-resistant lung cancer. In future studies, a gefitinib-resistant HCC827 cell line (HCC827-GR) should be established to examine the effect of YC-1 on enhancing the sensitivity of HCC827-GR to gefitinib.

In order to investigate the mechanism of the influence of the HIF-1 signaling pathway on the sensitivity of HCC827 cells to EGFR-TKIs, the correlation of p-Met levels in HCC827 cells with the activity of the HIF-1 signaling pathway was analyzed. A previous study indicated

that HIF-1 α is involved in the regulation of Met levels through EGFR. Additionally, EGFR regulation of Met levels in EGFR-TKI-sensitive cell lines occurs through the HIF-1 pathway in a hypoxia-independent manner (19,37). Nevertheless, in an EGFR-TKI-resistant cell line with a Met gene amplification, this regulation was lost, and if the overexpression of a constitutively active form of HIF-1 α occurred, this regulation was also lost (12). All these previous results indicate that the HIF-1 pathway, but not the EGFR pathway, may regulate the Met levels in EGFR-TKI-resistant cell lines caused by a Met gene amplification. In the present study, the p-Met levels in HCC827 cells were significantly positively correlated with the activity of the HIF-1 signaling pathway (Fig. 10; P <0.01; R^2 =0.978). This result is consistent with the results of previous research. Furthermore, previous research

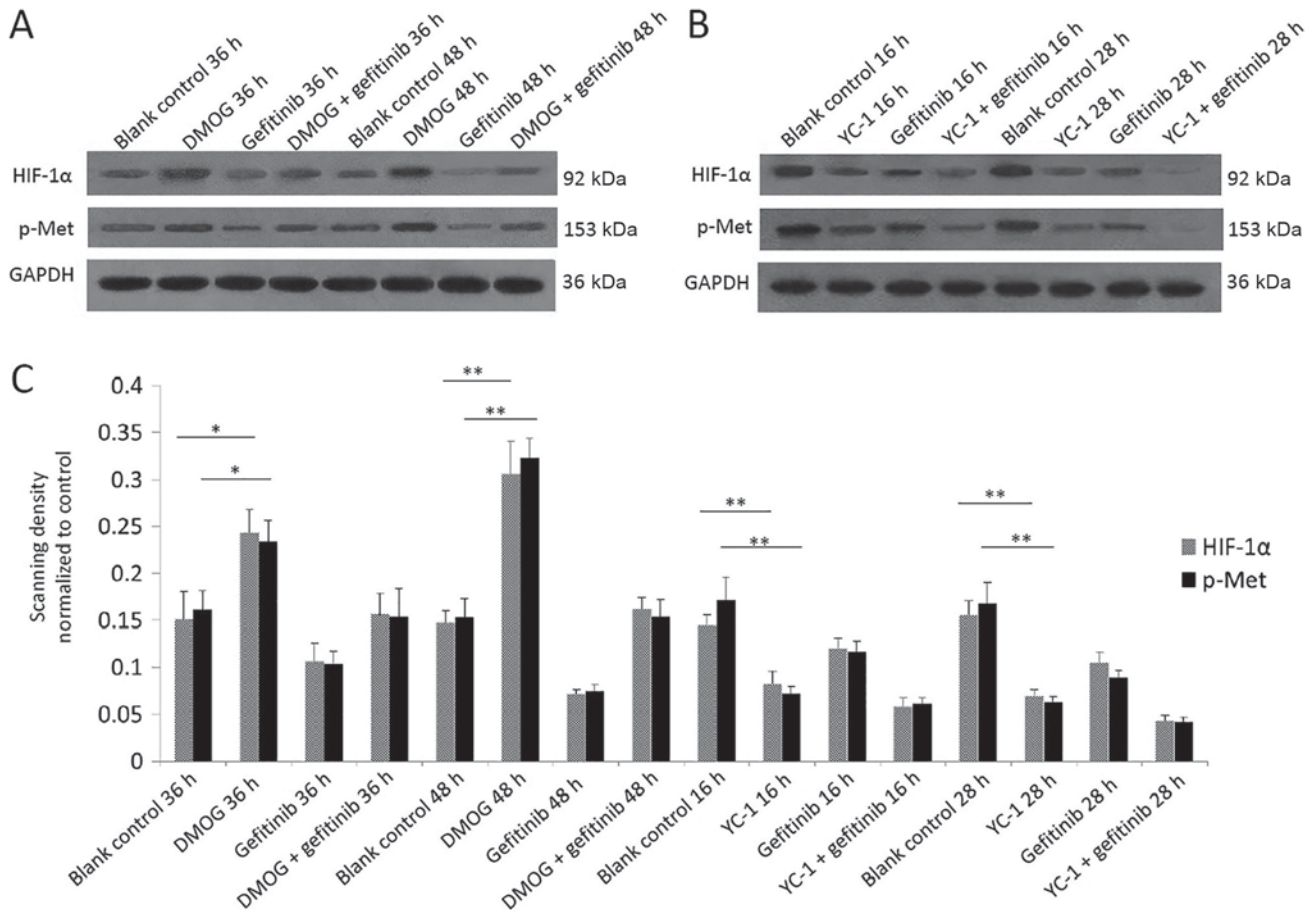


Figure 9. Expression of HIF-1 α and p-Met in HCC827 cells with different treatments. (A) Western blot analysis was performed to determine the HIF-1 α and p-Met levels in HCC827 cells with different treatments (blank control, DMOG, gefitinib, and DMOG and gefitinib combined for 36 and 48 h). Concentrations of DMOG and gefitinib used were 2 mM and 20 nM, respectively. (B) Western blot analysis was performed to evaluate the HIF-1 α and p-Met levels in HCC827 cells with different treatments (blank control, YC-1, gefitinib, and YC-1 and gefitinib combined for 16 and 28 h). Concentrations of YC-1 and gefitinib used were 40 μ M and 20 nM, respectively. (C) Quantified densitometric scanning of the blots. Error bars represent the mean \pm standard deviation from three independent experiments. *P<0.05 and **P<0.01. HIF-1 α , hypoxia-inducible factor-1; p-Met, phosphorylated hepatocyte growth factor receptor; DMOG, dimethylxalylglycine; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

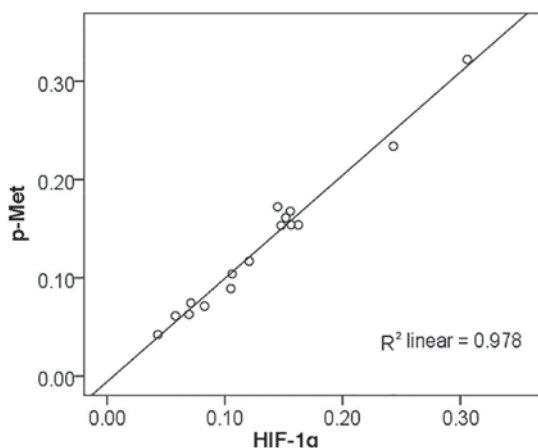


Figure 10. Correlation between HIF-1 α levels and p-Met levels of HCC827 cells with different treatments. Pearson's correlation analysis revealed that p-Met levels exerted a significant positive correlation with HIF-1 α levels in HCC827 cells with different treatments ($r^2=0.978$, P<0.01). HIF-1 α , hypoxia-inducible factor-1; p-Met, phosphorylated hepatocyte growth factor receptor.

demonstrated that Met amplification is the primary mechanism used for forming HCC827-GR cells through chronic

exposure to gefitinib (38-40). In future studies, the regulation of the HIF-1 pathway on Met levels in HCC827-GR cells should be observed, and the synergistic effect of YC-1 and gefitinib in HCC827-GR cells should be demonstrated.

Despite the requirement for further studies to investigate the synergistic effect of YC-1 and gefitinib in HCC827-GR cells, the present study reveals that the HIF-1 signaling pathway is able to influence the sensitivity of HCC827 cells to gefitinib, and that the associated mechanism may be the regulation of the HIF-1 pathway on p-Met. In conclusion, the HIF-1 pathway may be an attractive target for enhancing the sensitivity of NSCLC to EGFR-TKIs. This conclusion indicates the requirement for further research on the HIF-1 pathway as a target for overcoming or delaying acquired gefitinib resistance in NSCLC therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QJ analyzed and interpreted the data of the cell experiments, and was a major contributor in writing the manuscript. JZ performed the western blot assay and participated in the design of this research. XX performed the MTT assay. FH performed the colony formation assay. WX performed the cell migration assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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