Effects of hyperinsulinemia on acquired resistance to epidermal growth factor receptor-tyrosine kinase inhibitor via the PI3K/AKT pathway in non-small cell lung cancer cells *in vitro*

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Abstract. Patients with lung cancer harboring activating epidermal growth factor (EGFR) mutations and pre-existing diabetes have been demonstrated to exhibit poor responses to first-line EGFR-tyrosine kinase inhibitor (TKI) therapy. Strategies for the management of acquired resistance to EGFR-TKIs in patients with advanced non-small cell lung cancer (NSCLC) are urgently required. Only a limited number of studies have been published to date on the effects of insulin on EGFR-TKI resistance in NSCLC. Hence, the aim of the present study was to investigate the roles of hyperinsulinemia and hyperglycemia in mediating gefitinib resistance in NSCLC cells with activating EGFR mutations. In the present study, the HCC4006 cell line, which harbors EGFR mutations, was co-treated with gefitinib and long-acting insulin glargine. Whether hyperinsulinemia is able to mediate EGFR-TKI resistance in the NSCLC cell line harboring activating EGFR mutations was also investigated, and the possible underlying mechanisms responsible for these actions were explored. The inhibition of cell proliferation, and the potential mechanism of gefitinib resistance, were examined using an MTS proliferation assay and western blot analysis, and through the transfection of siRNAs. Whether the inhibition of AKT is able to overcome EGFR-TKI resistance induced by long-acting insulin was also investigated. The results obtained suggested that hyperinsulinemia induced by glargine upregulated NSCLC cell proliferation and survival, and induced gefitinib resistance. By contrast, the morphology and proliferation of the cells in a medium containing a 2-fold concentration of glucose were not significantly affected. Gefitinib resistance induced by hyperinsulinemia may have been mediated via the phosphoinositide 3-kinase (PI3K)/AKT pathway rather than the mitogen-activated protein kinase extracellular signal regulated kinase (MAPK/ERK) pathway. AKT serine/threonine kinase 1 knockdown by siRNA rescued the gefitinib resistance that was induced by hyperinsulinemia. In conclusion, hyperinsulinemia, but not hyperglycemia, was identified to cause the development of gefitinib resistance in NSCLC cells with activating EGFR mutations. However, additional studies are required to investigate strategies, such as co targeting hyperinsulinemia and the PI3K/AKT pathway, for overcoming EGFR-TKI resistance in patients with NSCLC.

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Abbreviations: NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; DM, diabetes mellitus

Key words: EGFR mutation, epidermal growth factor receptor-tyrosine kinase inhibitor, acquired resistance, lung cancer, diabetes, hyperinsulinemia

Introduction

Lung cancer is the leading cause of cancer associated mortality worldwide. The prognosis of patients with lung cancer is poor, and the overall 5-year survival rate is only 16% (1). One of the most crucial recent advances in the treatment of non-small cell lung cancer (NSCLC) has been the development of therapies targeting the epidermal growth factor receptor (EGFR). Tumors with activating EGFR mutations have been reported to be particularly sensitive to EGFR-tyrosine kinase inhibitors (TKIs) (2). EGFR-specific TKIs have been reported to be associated with improved progression-free survival rates in patients with EGFR mutation-positive NSCLC compared with

standard chemotherapies (3-5). However, acquired resistance to EGFR-specific TKIs may occur within 9-13 months (6); therefore, the mechanisms underlying acquired resistance warrant further investigation.

Patients with cancer who also have pre-existing diabetes mellitus (DM) exhibit higher mortality and recurrence rates following EGFR-TKI treatment compared with those without DM (7-9). DM is characterized by hyperglycemia, hyperinsulinemia, or insulin resistance. Additionally, a previous study reported that patients with lung cancer who harbored activating EGFR mutations and who had pre-existing DM, in conjunction with insulin-like growth factor 1 receptor (IGFR) overexpression, exhibited poor responses to first-line EGFR-TKI therapy (10). Furthermore, Ramalingam *et al* (11) demonstrated that cross-talk between IGFR and EGFR fulfills a critical role in tumorigenesis and in developing resistance to EGFR-TKIs in patients with NSCLC.

Insulin is able to activate insulin receptors and IGFR since they are structurally and functionally related heterotetrametric receptors that exhibit cross-talk (12). Exogenous insulin has been identified to mediate oncogenic effects. Hemkens *et al* (13) demonstrated that the insulin dose and long-acting insulin, for example glargine, are associated with a high risk of malignancy. Baglia *et al* (14) reported that insulin use may be associated with poor survival rates among patients with lung, breast, colorectal, or gastric cancer. However, to date, only a limited number of studies have been published on the effects of insulin on EGFR-TKI resistance in NSCLC.

In the present study, whether exogenous insulin mediates EGFR-TKI resistance in a selected NSCLC cell line harboring activating EGFR mutations, for example the HCC4006 cell line, was investigated, and the possible mechanisms underpinning these actions were elucidated. In addition, whether AKT inhibition is able to overcome EGFR-TKI resistance induced by long-acting insulin was also explored.

Materials and methods

Cell lines and cell cultures. The human NSCLC HCC4006 cell line, harboring the EGFR L747-E749 in-frame deletion in exon 19, was obtained from the American Type Culture Collection. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, HEPES, and 1.5 g/l sodium bicarbonate under an atmosphere of 5% CO_2 at 37˚C.

Reagents. Gefitinib (ZD 1839) was purchased from Sigma-Aldrich; Merck KGaA, and a 25 mM stock solution was prepared in dimethyl sulfoxide. Insulin glargine is a long-acting basal insulin analog. Glargine was purchased in its commercially packed form Sanofi S.A. at a concentration of 100 IU/ml, and a 600 μM stock solution was prepared in phosphate-buffered saline (PBS). Antibodies against phosphorylated (p)EGFR, total (t)EGFR, pIGFR, tIGFR, pAKT, tAKT, pERK, tERK, GAPDH and β-actin were used in the present study. The antibodies were diluted with 10% bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc.) and stored at -20˚C until use. The catalog numbers and dilutions of the antibodies used in the present study were as follows: AKT (cat. no. 4691S; 1:1,000); pAKT (cat. no. 4060S; 1:1,000); β-actin (cat. no. 8457S; 1:2,000); pEGFR (cat. no. 3777S; 1:1,000); EGFR (cat. no. 4267S; 1:1,000); pERK (cat. no. 4370S; 1:1,000); ERK (cat. no. 4695S; 1:1,000); GAPDH (cat. no. 2118S; 1:1,000); pIGFR (cat. no. 3024S; 1:300); and IGFR (cat. no. 3027S; 1:1,000). All antibodies were purchased from Cell Signaling Technology, Inc.

Proliferation assay. To determine the effects of gefitinib and glargine on NSCLC cell proliferation, HCC4006 NSCLC cells ($7.5x10^4$ cells/well) were plated onto 96-well plates. Following seeding for 24 h, the cells were treated with either RPMI-1640 culture medium (control) or with different concentrations of drugs, namely gefitinib (0-100 μM), glargine (1-100 nM), or both, in RPMI-1640 medium supplemented with 10% FBS. The cells in the growth medium were cultured for 24 h, and subsequently the cells were seeded into fresh media of 4 different compositions, according to the experimental treatment group concerned. A medium was prepared for the control group, without drug treatment. The cells in the other three media were treated with: i) Gefitinib only; ii) glargine only; and iii) gefitinib + glargine, respectively. The cells of the control group and the other three drug treatment groups were subsequently cultured for a further 24 h. At the end of the treatment period, cell proliferation was measured using the MTS assay (BioVision, Inc.), which is based on the reduction of the MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. The drug concentration values required to inhibit cell proliferation by 50% (IC_{50}) were determined from the dose-response curves for each treatment by using interpolation. Between 5 and 8 replicate wells were used for each analysis, and at least 3 independent experiments were performed. Data from replicate wells were used to determine the mean number of viable cells. The mean number of viable cells in each group were reported with 95% confidence intervals. To determine the effect of the combined drug treatments, any potentiation was estimated by multiplying the percentages of the cells remaining (% proliferation) in each treatment.

Western blot analysis. Following each treatment, the cells were washed 3 times with ice-cold PBS. Subsequently, cells were directly lysed with 1X Laemmli buffer [comprising 2% sodium dodecyl sulfate (SDS), 5 mM dithiothreitol (DTT), 10% glycerol, 0.002% bromophenol blue, and 63 mM Tris-HCl (pH 6.8)], and heated at 98˚C for 5 min. The total protein concentrations were determined using the BCA protein assay. Samples containing 20-30 μg protein were then separated on an SDS-PAGE by using 6-15% gels. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes (cat. no. 1620177; Bio-Rad Laboratories, Inc.). The membranes were briefly activated in methanol and blocked with 5% milk in TBST buffer (consisting of 20 mM Tris, 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature, followed by overnight incubation at 4˚C with antibodies specific to the target proteins. Next, the membranes were washed 3 times for 10 min with TBST and hybridized with goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugated secondary antibodies (cat. no. sc-2004; Santa Cruz Biotechnology) with 1:5,000 dilution (in TBST containing 5% milk) at room temperature for a further 1 h. After washing

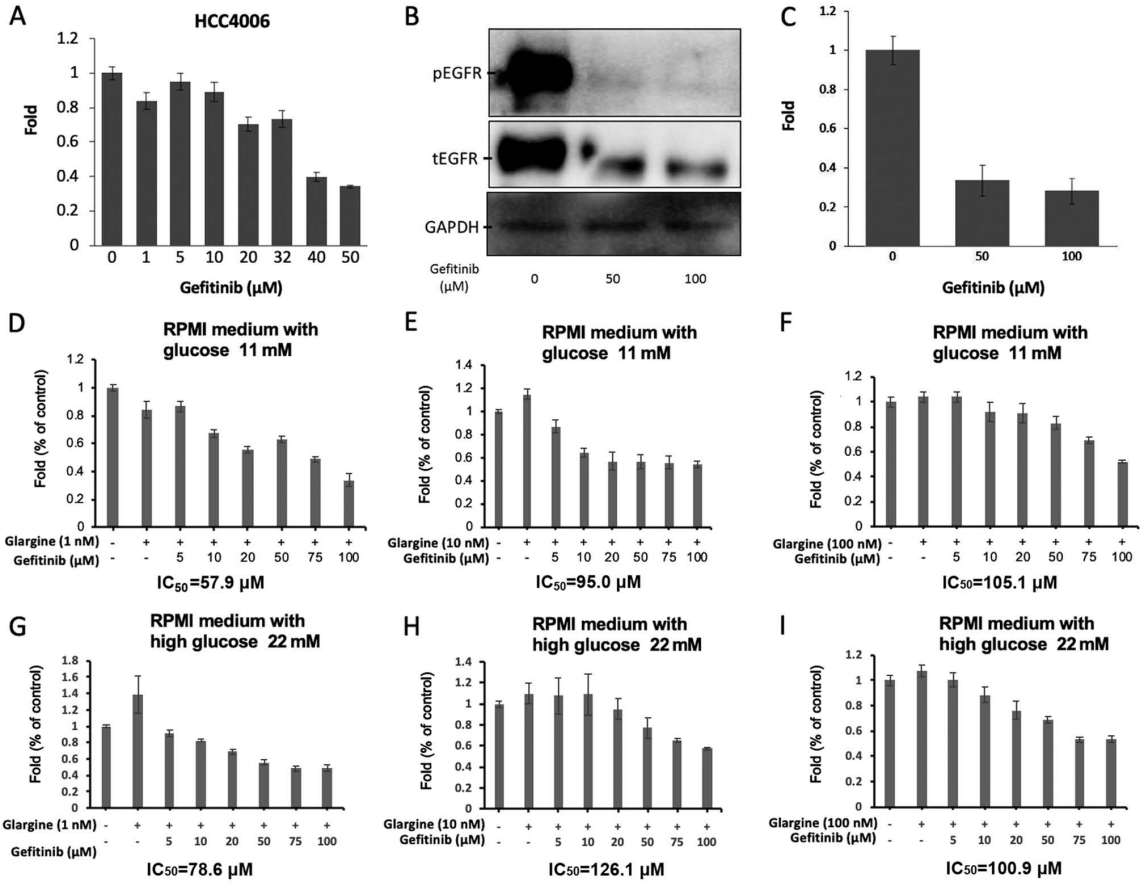

Figure 1. Effect of gefitinib treatment on NSCLC cells harboring EGFR mutations. (A) HCC4006 cells were treated with gefitinib for 24 h, and cell survival was measured using an MTS proliferation assay. (B) HCC4006 cells were treated with the indicated concentrations of gefitinib (0-50 nM) for 24 h. The cells were then harvested, and equal amounts of protein lysate were subjected to western blot analysis to determine the levels of pEGFR and EGFR expression. The level of GAPDH was used as a reference for the lysate protein loading control of the cell line. (C) Quantification of the results of the western blot analysis is shown in the bar graph. (D-F) HCC4006 cells were treated with gefitinib and different concentrations of glargine in RPMI medium with 11 mM glucose, and cell survival was measured using an MTS proliferation assay. (G-I) HCC4006 cells were treated with gefitinib and different concentrations of glargine in RPMI medium with 22 mM high-glucose, and cell survival was measured using the MTS proliferation assay. NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; p, phosphorylated; t, total; IC_{50}, half-maximal inhibitory concentration.

3 times for 5 min each in TBST, the bands on the membranes were visualized by adding an enhanced chemiluminescence substrate (cat. no. RPN2235; GE Healthcare). All images were recorded using a ChemiDoc gel imaging system (Bio-Rad Laboratories, Inc.), and densitometric analysis was conducted using Image Lab software version 6.0.1 (Bio-Rad Laboratories, Inc.). Internal controls (GAPDH) were used to normalize the target protein, and the mean quantities were represented graphically.

siRNA transfection. To determine whether AKT serine/threonine kinase 1 (AKT1) was mainly responsible for the development of gefitinib resistance, the proliferation of the HCC4006 cells was examined following knockdown of AKT1. For siRNA transfection, HCC4006 cells were transfected with siRNA oligo (5'-AAAUCCAGACUCUUUCGAU-3') targeting EGFR 3'-UTR with the Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Inc.) and used for experiments 48 h after transfection. siRNAs against Akt1 (5'-GACAAGGACGGGCACAUUA-3') were purchased from GE Healthcare Dharmacon. Briefly, prior to cell seeding, the final concentration of human AKT1 siRNA was calculated to be 10 nM in a mixture containing 2X Lipofectamine RNAiMAX. The cells were incubated at room temperature for 15 min. Next, the cells were trypsinized, counted, and mixed with either the siRNA knockdown group solution or control group solution and seeded 10,000 cells per well on a 96-well plate for the MTS assay or 24-well plate for western blot analysis, as aforementioned.

Statistical analysis. Each experiment was replicated at least three times. Data were expressed as mean ± SEM. Data were analyzed using a one-way analysis of variance (ANOVA), and Tukey's post hoc test. Two-sided $P<0.05$ were considered to indicate a statistically significant difference. All data were analyzed using SigmaPlot 13 (v13.0.0.83; Systat Software, Inc.).

Results

Effect of gefitinib treatment on NSCLC cells harboring EGFR mutations. The effects of gefitinib on cell proliferation were assessed using an MTS assay and the basal levels of EGFR and pEGFR in HCC4006 cells were measured. As shown in Fig. 1, NSCLC cells harboring the EGFR mutation in exon 19 responded to treatment with gefitinib. HCC4006 cells were

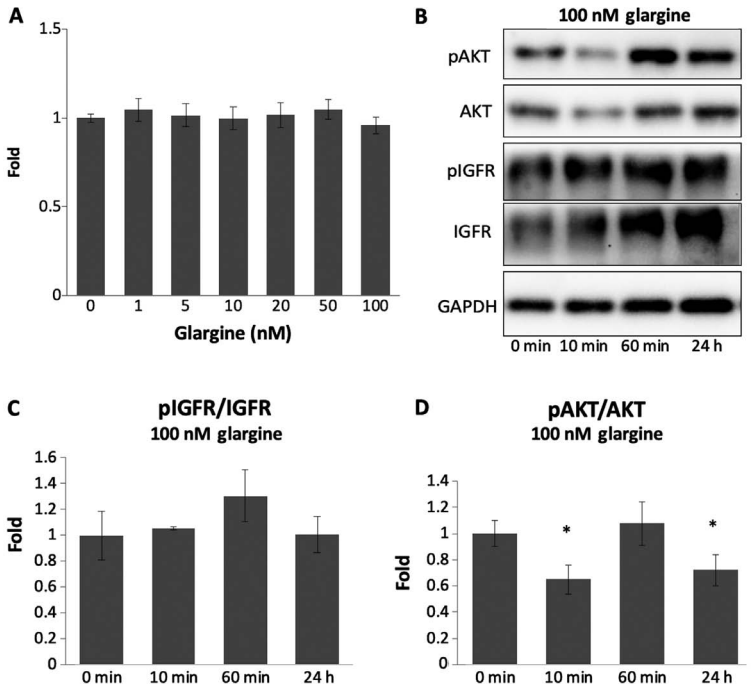

Figure 2. Long-acting insulin glargine did not significantly affect NSCLC cells. (A) HCC4006 cells were initially plated in 96-well plates and grown in experimental medium containing different concentrations of long-acting insulin glargine for 24 h. Cell survival was measured using the MTS proliferation assay. Quantification of the results obtained from the MTS proliferation assay is shown in the bar graph. The vertical bars indicate fold-change compared with the control group. (B) Western blot analysis of pIGFR, IGFR, pAKT, and AKT was performed in HCC4006 cells treated with 100 nM glargine for different time periods (0, 10 and 60 min, and 24 h). (C and D) Quantification of the data of the western blot analysis of pIGFR, IGFR, pAKT and AKT is shown using bar graphs. The vertical bars indicate the fold-change compared with the control group. *P<0.05 vs. control group using a one-way ANOVA with Tukey's post hoc test. NSCLC, non-small cell lung cancer; p, phosphorylated; IGFR, insulin-like growth factor receptor.

sensitive to gefitinib, with an IC_{50} value of 39.42 μM (Fig. 1A). Subsequently, the tyrosine kinase activity of EGFR was demonstrated to be inhibited in a dose-dependent manner, depending on the dose of EGFR-TKI, in the HCC4006 cell line. Specifically, in the western blot analysis assay, the protein level of pEGFR in HCC4006 cells decreased considerably following treatment with gefitinib (Fig. 1B). The HCC4006 cells expressed high levels of both EGFR and pEGFR. However, the expression levels of tEGFR and pEGFR were suppressed in HCC4006 cells treated with 50 and 100 μM gefitinib for 72 h in a dose-dependent manner (Fig. 1C). When HCC4006 cells were treated with gefitinib and different concentrations of glargine in RPMI medium with 11 mM glucose, the IC_{50} values of gefitinib were 57.9 μM with 1 nM glargine (Fig. 1D), 95.0 μM with 10 nM glargine (Fig. 1E), and 105.1 μM with 100 nM glargine (Fig. 1F). Meanwhile, the IC_{50} values of gefitinib were 78.6 μM with 1 nM glargine (Fig. 1G), 126.1 μM with 10 nM glargine (Fig. 1H), and 100.9 μM with 100 nM glargine (Fig. 1I), when HCC4006 cells were treated with gefitinib and different concentrations of glargine in 22 mM high-glucose of RPMI medium.

Long-acting insulin glargine did not significantly affect the NSCLC cells. Initially, the degradation of porcine insulin in HCC4006 cells was investigated. As shown in Fig. S1, the expression of pAKT/tAKT decreased at 60 min and 24 h after the addition of porcine insulin, which indicated porcine insulin might not be active for 24 h. Therefore, glargine (long-acting insulin) was selected in this study. To determine the roles of glargine in the development of gefitinib resistance, the effects of glargine alone on the proliferation of HCC4006 cells were subsequently investigated. As shown in Fig. 2, glargine treatment did not exert a significant effect on the proliferation of HCC4006 cells, even though HCC4006 cells were treated with different concentrations of glargine (from 0 to 100 nM). No significant differences in cell proliferation were observed following the application of different doses of glargine (Fig. 2A). Subsequently, in HCC4006 cells treated with 100 nM glargine for different time intervals, the levels of pIGFR/tIGFR and pAKT/tAKT were quantified through western blot analysis (Fig. 2B). The levels of pIGFR/tIGFR did not change significantly (Fig. 2C), whereas the level of pAKT/tAKT was significantly decreased following treatment time periods of 10 min and 24 h (Fig. 2D).

Mechanism of hyperinsulinemia in the development of gefitinib resistance. Subsequently, whether glargine was able to affect the efficiency of action of gefitinib, ultimately leading to gefitinib resistance, was investigated in HCC4006 cells. As shown in Fig. 3A, HCC4006 cells were cultured in media containing different concentrations of glargine and a fixed concentration of gefitinib (at the IC_{50} concentration of 32 μM) for 24 h. The proliferation of HCC4006 cells decreased significantly following treatment with gefitinib alone. However, the MTS assay revealed that the proliferation of HCC4006 cells co-treated with gefitinib and different concentrations of glargine (0-100 nM) led to a marked increase in cell proliferation compared with the control cells. Glargine (5-100 nM) caused a significant increase in the fold-change in the MTS assay (50-80%), which indicated that glargine increased cell

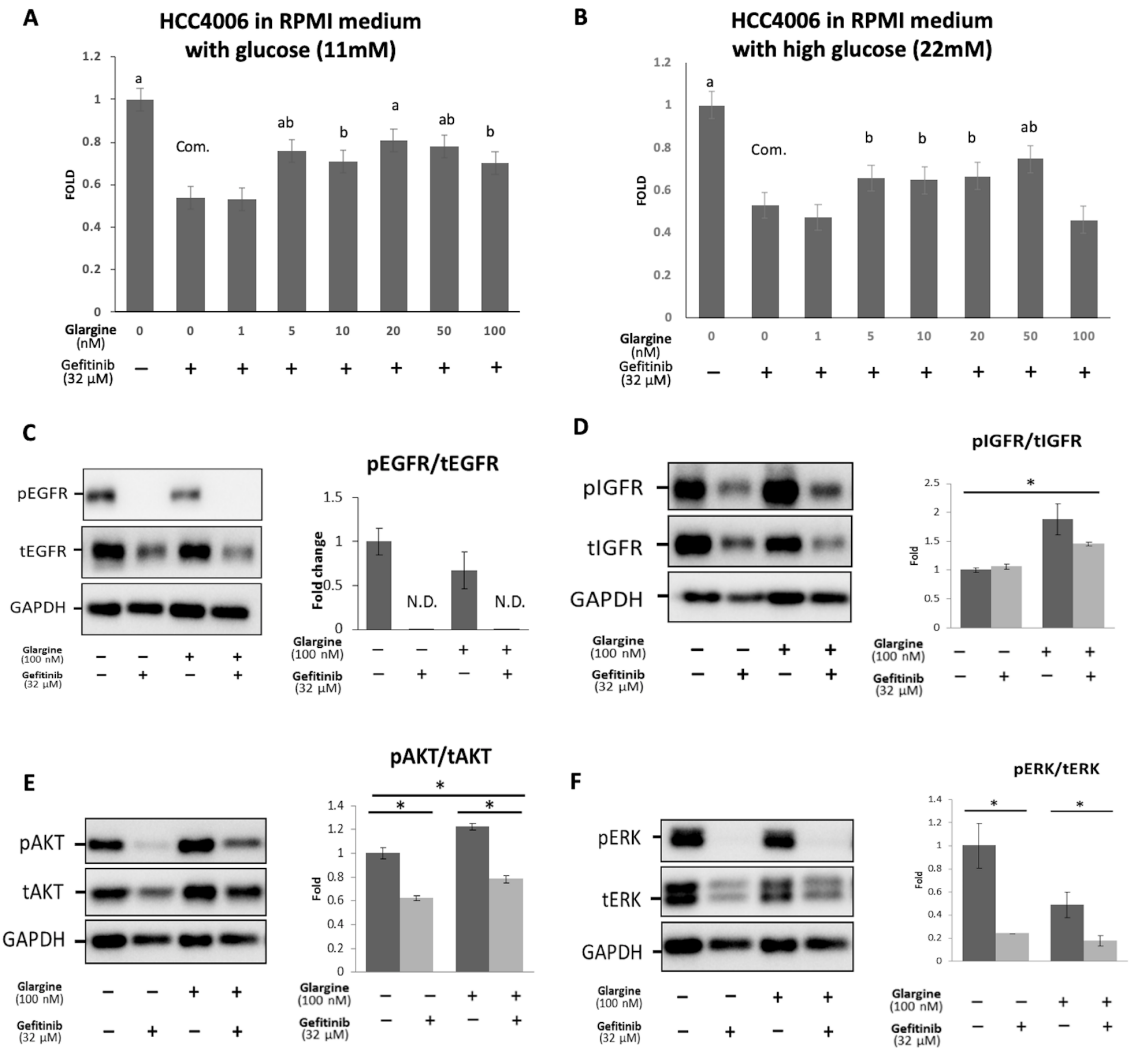

Figure 3. Mechanism of hyperinsulinemia induced by glargine in the development of gefitinib resistance. (A) HCC4006 cells were cultured in regular RPMI medium containing 11 mM glucose. (B) The cells were cultured in RPMI medium containing a 2-fold concentration of glucose (22 mM). HCC4006 cells were treated with different concentrations of glargine, and a fixed concentration of gefitinib (IC_{50} value, 32 μM) for 24 h. Cell survival was measured using an MTS proliferation assay. The vertical bars indicate a fold-change compared with the control group. The eight groups were analyzed in a single one-way ANOVA with Tukey's post hoc test. $^{a}P<0.001$, $^{ab}P<0.01$, and $^{b}P<0.05$ vs. gefitinib 32 μM without glargine group. (C-F) HCC4006 cells were treated with 32 μM gefitinib, 100 nM glargine, or both gefitinib and glargine for 24 h. Western blot analysis was performed to investigate the protein expression levels of pEGFR, EGFR, pIGFR, IGFR, pAKT, AKT, pERK and ERK. $^{*}P<0.05$ vs. control group using a one-way ANOVA with Tukey's post hoc test. EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; IGFR, insulin-like growth factor receptor; p, phosphorylated; t, total; com., comparison group; N.D., none detected.

proliferation in a dose-dependent manner. Therefore, we concluded that the hyperinsulinemia induced by glargine was able to affect the efficiency of gefitinib action, and furthermore, may induce gefitinib resistance.

The aim of the present study was not only focused on the effects of hyperinsulinemia, but the interaction between hyperglycemia and hyperinsulinemia was also explored, as the role of glucose in the development of gefitinib resistance could not be excluded. As shown in Fig. 3B, the RPMI-1640 medium of altered composition (22 mM), containing a 2-fold concentration of glucose compared with the original concentration (11 mM), was used. HCC4006 cells were cultured in high-glucose RPMI-1640 medium (22 mM) for 3 passages. However, the morphology and proliferation of cells grown in high-glucose RPMI-1640 medium (22 mM) and those grown in the original medium containing 11 mM glucose did not differ significantly.

To determine the role of hyperinsulinemia in the development of gefitinib resistance, the expression of EGFR, pEGFR, IGFR, and pIGFR, and their downstream factors, such as AKT, pAKT, extracellular signal-regulated kinase (ERK), and pERK, were evaluated to elucidate the mechanisms of gefitinib resistance. As shown in Fig. 3C, HCC4006 cells were treated for 24 h with 32 μM gefitinib alone, 100 nM glargine alone, or 32 μM gefitinib + 100 nM glargine. These experiments revealed that the phosphorylation of EGFR was significantly suppressed by either gefitinib or co-treatment with gefitinib and glargine. The ratio of pEGFR/tEGFR was not detectable in the cells treated with gefitinib or co-treated with gefitinib and glargine. As shown in Fig. 3D, no significant difference in the pIGFR level was observed between control and gefitinib-treated HCC4006 cells, which indicated that the IGFR pathway could operate independently. The pIGFR level was

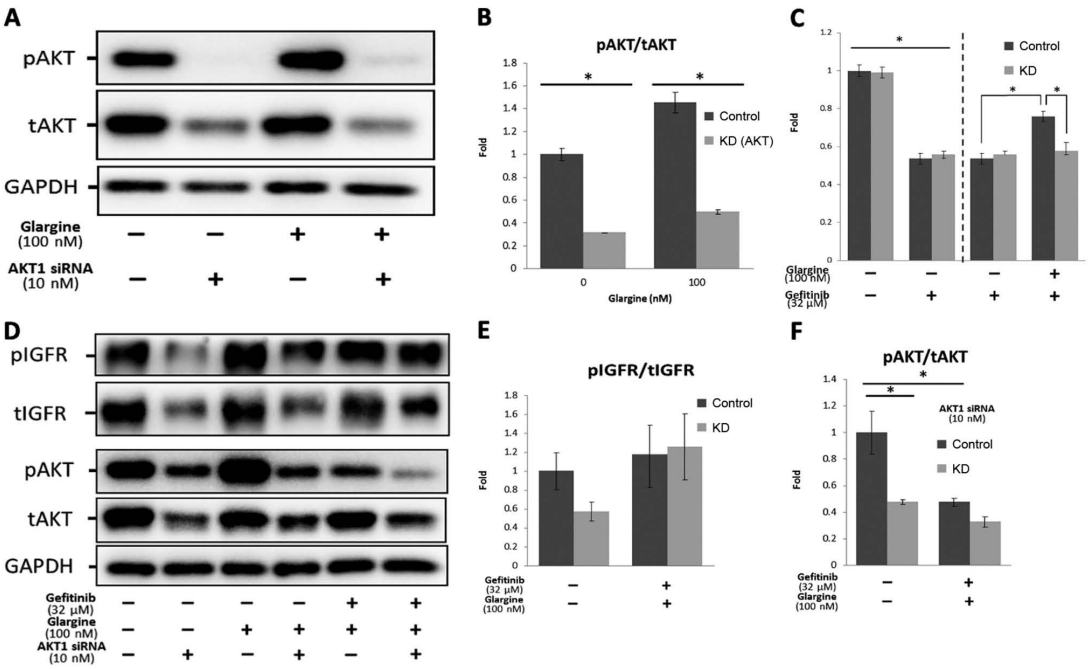

Figure 4. AKT1 knockdown through siRNA transfection was able to rescue gefitinib resistance induced by glargine. (A and B) HCC4006 cells were treated with or without 10 nM AKT1 siRNA, and then treated with or without 100 nM glargine for 24 h, and the results are shown in the representative western blots and in the bar graphs. (C-F) HCC4006 cells were treated with or without 10 nM AKT1 siRNA, and subsequently treated with 32 μM gefitinib, 100 nM glargine, or both. Results are shown in the representative western blots and the bar graphs. (C) Cell survival was measured using an MTS proliferation assay. The vertical bars indicate the fold-change compared with the control group. (D-F) Quantification of the results of the western blot analysis of pAKT, AKT, pIGFR and IGFR is shown. The vertical bars indicate the fold-change compared with the control group. *P<0.05 vs. control group using a one-way ANOVA with Tukey's post hoc test. IGFR, insulin-like growth factor receptor; p, phosphorylated; t, total; siRNA, small interfering RNA; KD, knockdown; IC_{50}, half-maximal inhibitory concentration.

significantly increased in the glargine-treated group compared with the gefitinib-treated group, thereby suggesting that the hyperinsulinemia induced by glargine was able to switch on the IGFR pathway, which may represent a potential route for gefitinib resistance.

To determine downstream pathway factors, AKT, pAKT, ERK, and pERK were further investigated. As shown in Fig. 3E, pAKT expression was markedly suppressed in the gefitinib-treated cells. However, among the cells treated with glargine or glargine and gefitinib, pAKT expression was notably increased (P<0.01), which suggested that hyperinsulinemia induced by glargine may switch on the IGFR pathway, with cross-talk compensation via the AKT pathway. Therefore, cell proliferation was observed, even when gefitinib inhibited the EGFR downstream pathway. As shown in Fig. 3F, the pERK expression level was significantly decreased in the gefitinib treatment group (P<0.05). However, the pERK level was completely suppressed even when glargine and gefitinib were added simultaneously, which was opposite to the effects on pAKT expression. This result indicated that gefitinib resistance may not have resulted from the mitogen-activated protein kinase (MAPK)/ERK pathway.

AKT1 knockdown by siRNA rescued gefitinib resistance induced by glargine. Subsequently, AKT was knocked down by transfecting the cells with the siRNA of AKT1 to confirm that gefitinib resistance resulted from the AKT pathway induced by glargine. First, the efficiency of AKT knockdown was investigated using an MTS assay and western blot analysis (Fig. 4A and B). Next, 10 nM AKT1 siRNA was added, and the cells were incubated for 24 h. Then, 100 nM glargine, show to activate the AKT pathway in Fig. 3E, was added. As shown in Fig. 4A and B, pAKT expression in the knockdown group was significantly suppressed, and tAKT expression was successfully knocked down by AKT siRNA. Furthermore, AKT siRNA did not exert any effect on glargine treatment. When glargine was subsequently added, pAKT expression was not activated. As shown in Fig. S2A and B, the expression of IGFR1, insulin receptor, and pIGFR were not affected by the knockdown of AKT. When 100 nM glargine was added, pIGFR was activated (P<0.05) in both control and the AKT knockdown groups. However, the expression of pIGFR did not significantly change between the control and the AKT knockdown groups regardless of the addition of glargine, which indicated that pIGFR activation was independent of transfection with AKT siRNA. The function of gefitinib, which exerted appropriate effects in the control and knockdown groups, was subsequently investigated. Furthermore, the IC_{50} values comparing the siRNA knockdown experiment in Fig. S2C (37 μM) and the control group in Fig. 1A (39 μM) were not significantly different. Cell proliferation was not significantly increased in the knockdown group compared with in the control group when cells were cotreated with 32 μM gefitinib and 100 nM glargine (Fig. 4C).

To examine the pathway markers, the expression of pIGFR/IGFR, pAKT/AKT, and pERK/ERK was then investigated. As shown in Fig. 4D and F, the expression levels of pAKT and tAKT were significantly decreased in the knockdown group compared with the control group, and no differences were observed between the knockdown and the co-treatment (gefitinib and glargine) groups. When HCC4006

cells were co-treated with gefitinib and glargine, the expression level of pAKT remained unchanged. No significant differences were observed between the control and knockout groups co-treated with gefitinib and glargine, suggesting that AKT had been successfully knocked down so that the effects of hyperinsulinemia induced by glargine could not be mediated via the AKT pathway.

As shown in Fig. 4D and E, no significant differences in the expression levels of pIGFR/IGFR were observed between the control and knockdown group, irrespective of whether co-treatment with gefitinib and glargine was performed (P=0.134) or not (P=0.516). This result may indicate that the IGFR pathway, which is normally activated, was not suppressed by gefitinib.

As shown in Fig. S2D and E, the levels of pERK were significantly inhibited in the AKT knockdown group (P<0.01), whereas no inhibition was observed in the cells co-treated with gefitinib and glargine. However, no significant differences were observed between the AKT knockdown group (co-treated with gefitinib and glargine) and the control group. This could indicate that gefitinib resistance induced by hyperinsulinemia is not directly affected by the ERK pathway.

Discussion

In the present study, HCC4006 cells were demonstrated to be sensitive to gefitinib. Co-treatment with glargine (0-100 nM) and gefitinib induced gefitinib resistance in HCC4006 cells in a dose-dependent manner. To elucidate the mechanism underlying gefitinib resistance induced by glargine, western blot analysis was performed to investigate the participating signaling pathways. According to the western blot analysis results, insulin (glargine) increased the phosphorylation levels of IGFR and AKT excluding ERK, suggesting that glargine may primarily reactivate the PI3K/AKT pathway, which is associated with cell proliferation, instead of the MAPK/ERK pathway, which directly affects DNA transcription in the nucleus. Furthermore, via knocking down the expression of AKT1 through siRNA transfection, these experiments were able to further establish the role of AKT in the development of gefitinib resistance. The MTS assay results revealed that levels of cell proliferation were decreased in the AKT knockdown cells compared with the cells that were not transfected, suggesting that AKT is essential for the development of resistance.

Patients with NSCLC and pre-existing DM have been demonstrated to exhibit shorter survival rates compared with those without DM (15). DM is a metabolic disorder characterized by hyperglycemia and hyperinsulinemia. IGFR has been reported to be a negative biomarker of resistance to the TKI gefitinib in NSCLC cell lines and patients with NSCLC (16). Therefore, it was possible to hypothesize that insulin may serve an essential role in the development of gefitinib resistance. The present study has revealed that long-acting insulin glargine was able to decrease the efficiency of gefitinib action in HCC4006 cells co-treated with gefitinib and glargine. Furthermore, the reason why the long-acting insulin glargine was selected in preference to porcine insulin was due to the difference in their duration of action. The western blot analysis data indicated that hyperinsulinemia induced by glargine led to a continuation of the activation of the insulin pathway and suppression of the level of pAKT for >24 h. Conversely, the activity of porcine insulin lasted only for 2-4 h in Fig. S1. Therefore, the long-acting insulin glargine was selected for the present study.

Hyperinsulinemia and hyperglycemia are 2 essential features of DM. Therefore, the present study aimed to clarify the role not only of high insulin concentrations but also of high-glucose concentrations in terms of the development of gefitinib resistance. High-glucose RPMI-1640 medium (22 mM) contained twice the glucose concentration of regular RPMI-1640 medium (11 mM). HCC4006 cells were passaged 3 times in high-glucose medium (22 mM), and it was confirmed that the morphology and proliferation of the cells were unaffected by high-glucose medium compared with that of HCC4006 cells cultured in regular RPMI-1640 medium (11 mM). The results of the present study demonstrated that the development of gefitinib resistance may be independent of high-glucose concentrations and may predominantly be affected by the activation of insulin.

In the present study, hyperinsulinemia induced gefitinib resistance in HCC4006 cells, and this resistance was primarily affected by activation of the PI3K/AKT pathway rather than the MAPK/ERK pathway. Tyrosine kinase receptors, such as IGFR or EGFR, were shown to activate the PI3K/AKT and MAPK/ERK pathways. The dysregulation of the PI3K/AKT pathway has been reported to frequently occur in NSCLC (17). Cross-talk between EGFR and IGFR can occur through interaction between shared downstream components of the activated receptors. Therefore, multilayered cross-talk and involvement of shared components of the signaling pathways may lead to acquired resistance to EGFR-TKI therapy in the treatment of cancer (18). The PI3K/AKT pathway fulfills an integral part in intracellular signal transduction to promote metabolism, proliferation, apoptosis, and angiogenesis (19-21). Activation of the PI3K/AKT pathway has been reported to cause resistance to various anticancer therapies, including chemotherapy and TKIs (22-24). Certain studies have shown that the PI3K/AKT pathway exerts a critical role in the development of resistance to TKIs (24,25). In the present study, the low expression levels of pERK and tERK, despite simultaneous glargine treatment, may have resulted from the high dose of gefitinib (32 μM) used.

The gefitinib dose employed in the present study was 32 μM, which was increased compared with that used in other studies (26,27). As the duration of glargine action was an important factor in these experiments, HCC4006 cells were exposed to drug treatment for only 24 h prior to subsequent harvesting of the cells. This duration of treatment was shorter compared with that employed in other studies (72 h) (26,27). Owing to the short treatment time in the present study (only 24 h), it was necessary to use a high gefitinib concentration to achieve the appropriate IC_{50} value. However, to exclude the possibility that the increased dose of gefitinib might have exerted adverse effects on the morphology of the HCC4006 cells, the cells were treated with the higher concentration of gefitinib for 72 h, and at the end of the treatment period, normal cell morphology was still observed.

Acquired resistance to EGFR-TKI is a 'Gordian knot' to be tackled, especially in patients with NSCLC accompanied by DM. The present study investigated whether exogenous insulin or a high-glucose concentration could facilitate the

development of resistance to EGFR-TKI in the NSCLC cell line (HCC4006). The results obtained revealed that treatment with long-acting exogenous insulin, instead of a high concentration of glucose, could lead to gefitinib resistance in NSCLC cells with activating EGFR mutations. In addition, the mechanism of gefitinib resistance involved reactivation of the PI3K/AKT pathway, rather than the MAPK/ERK pathway. Furthermore, it was observed that the gefitinib resistance could be circumvented by knocking down the expression of AKT1 through siRNA transfection. However, in spite of these advances in our knowledge, additional research is warranted, both to investigate the mechanism in a xenograft mouse model and to examine the effects of co-targeting exposure to exogenous insulin and the reactivation of the PI3K/AKT pathway to overcome EGFR-TKI resistance in NSCLC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HYC, CMC, SPY, DSJ, LSW, HCL and CHC conceived and designed the study. HYC, CMC and SPY performed the experiments and the statistical analysis. HYC, CMC and SPY acquired, analyzed and interpreted the data. HYC and CMC wrote the original manuscript. SPY, DSJ, LSW, HCL and CHC revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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