Metformin sensitizes EGFR-TKI-resistant human lung cancer cells in vitro and in vivo through inhibition of IL-6 signaling and EMT reversal

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Statement of translational relevance

Although NSCLC patients with EGFR mutations show an excellent initial response to EGFR-TKIs, their acquired resistance inevitably arises and has no effective treatment yet. In this study, we investigated whether metformin could reverse TKI resistance in TKI-resistant cell lines and xenograft models. Our data suggest that metformin could effectively overcome TKI resistance both \textit{in vitro} and \textit{in vivo} by inhibiting IL-6 signaling pathway and reverting EMT. Thus, in NSCLC patients with EGFR mutations, metformin might be used in combination with TKIs to delay or overcome TKI resistance. We have already successfully started a clinical trial (http://www.clinicaltrials.gov/ct2/show/NCT01864681) to observe the benefits of metformin in combination with gefitinib to treat advanced NSCLC patients with EGFR mutations. The current study provides the pre-clinical evidence that metformin has promising potential to be developed as a novel anti-cancer agent.
ABSTRACT

Purpose: The epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have become a standard therapy in patients with EGFR-activating mutations. Unfortunately, acquired resistance eventually limits the clinical effects and application of EGFR-TKIs. Studies have shown that suppression of EMT and IL-6/STAT3 pathway may abrogate this acquired mechanism of drug resistance of TKIs. This study aims to investigate the effect of metformin on sensitizing EGFR-TKI-resistant human lung cancer cells in vitro and in vivo through inhibition of IL-6 signaling and EMT reversal.

Experimental design: The effect of metformin on reversing TKI resistance was examined in vitro and in vivo using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium(MTT), BrdU incorporation assay, invasion assay, flow cytometry analysis, immunostaining, Western blot analysis and xenograft implantation.

Results: In this study, metformin, a widely used antidiabetic agent, effectively increased the sensitivity of TKI-resistant lung cancer cells to erlotinib or gefitinib. Metformin reversed EMT and decreased IL-6 signaling activation in TKI-resistant cells, while adding IL-6 to those cells bypassed the anti-TKI-resistance effect of metformin. Furthermore, overexpression or addition of IL-6 to TKI-sensitive cells induced TKI resistance, which could be overcome by metformin. Finally, metformin-based combinatorial therapy effectively blocked tumor growth in xenografts with TKI-resistant cancer cells, which was associated with decreased IL-6 secretion and expression, EMT reversal and decreased IL-6 signaling activation in vivo.

Conclusion: Metformin, generally considered non-toxic and remarkably inexpensive, might be used in combination with TKIs in NSCLC patients harboring EGFR mutations to overcome TKI resistance and prolong survival.
INTRODUCTION

Reversible small-molecule epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), including gefitinib (Iressa) and erlotinib (Tarceva), have shown dramatic therapeutic efficacy in non-small-cell lung cancer (NSCLC) patients with EGFR-activating mutations, and have been recommended as the standard first-line therapy in these patients (1, 2). However, despite excellent initial clinical responses, nearly all responding patients eventually develop drug resistance after a median period of about 10 months (3). Thus, innovative treatment strategies are urgently needed to overcome therapeutic resistance to EGFR-TKIs to improve the survival of NSCLC patients.

Molecular mechanisms underlying acquired TKI resistance are still not fully understood. Two principal mechanisms accounting for approximately 50% of acquired resistance to TKIs in lung cancer are secondary mutations of a threonine-to-methionine substitution at amino acid position 790 (T790M) of EGFR and amplification of the N-Methyl-N’-nitro-N-nitroso-guanidine (MNNG) HOS transforming gene (MET) oncogene (3, 4). Various other molecular mechanisms are also involved, including epithelial mesenchymal transition (EMT). EMT is a process during which cells undergo morphological changes from epithelial phenotype to mesenchymal phenotype, resulting in enhanced motility and increased invasion, proliferation and metastasis of cancer cells (5). EMT has been associated with the sensitivity not only to conventional chemotherapies in several types of cancer (6, 7), but to EGFR-TKIs in lung cancer cells, xenografts and patients (8). Targeting EMT may reverse or prevent acquisition of therapeutic resistance to EGFR inhibitors (9). TGF-β is a major driving force of the EMT genetic program (5). It was reported that TGF-β could induce IL-6 axis activation, further resulting in EMT and resistance to reversible TKI in lung cancer cells, while inhibiting IL-6/STAT3 signaling could restore TKI sensitivity (10). In NSCLC with T790M mutations, activation of IL-6R/JAK1/STAT3 signaling...
could induce resistance to irreversible EGFR inhibitors (11). IL-6/STAT3 signaling may be a potential therapeutic target for enhancing the efficacy of EGFR-TKIs. Therefore, reversing EMT and decreasing IL-6 signaling activation may be an effective way to improve the response to EGFR-TKI treatment.

Metformin is a widely used and well tolerated drug for diabetes and has arisen keen interest as a potential anticancer agent ever since the report of the clinical evidence that the cancer risk and mortality are reduced in diabetics receiving metformin (12). Metformin exerts remarkable antitumor properties in tumor cells and mouse models. It strongly inhibited the growth of lung cancer cells (13), and its combination with metformin and classical chemotherapeutic agents, including paclitaxel, cisplatin or doxorubicin, significantly suppressed breast tumor growth and prolonged remission in a xenograft model (14). Interestingly, metformin exposure significantly impeded the TGF-β-promoted EMT process (15), decreased IL-6 secretion, and suppressed STAT3 activity (16), suggesting that metformin may overcome TKI resistance by reversing EMT and inhibiting IL-6 signaling pathways. One recent study reported that metformin in combination with gefitinib significantly enhanced the efficacy of targeted therapy (17). However, the mechanism underlying this effect remains unclear.

Here we show that metformin in combination with gefitinib or erlotinib has a synergistic inhibitory effect on the proliferation, migration and invasion of cell lines resistant to TKIs. The effect of metformin against TKI resistance is attributed to its ability to reverse EMT and decrease IL-6 signaling activation. In addition, this combinational therapy reduces tumor masses much more effectively than gefitinib or metformin alone in a xenograft mouse model, and this synergistic interaction is associated with the ability of metformin to decrease the activation of STAT3 and AKT and reverse EMT. We thus provide rationale and experimental evidence for the combined use of metformin and TKIs to overcome TKI resistance in NSCLC patients with EGFR mutations.
MATERIALS AND METHODS

Cell lines and reagents

Gefitinib (Iressa) was purchased from Tocris Bioscience and erlotinib (Tarceva) from Cayman Chemical. Both drugs were prepared in dimethyl sulfoxide (DMSO) to obtain a stock solution of 10 mM. Metformin (Sigma) was dissolved in deionized water and stored at -20 °C. The recombinant human IL-6 (rhIL-6) was purchased from PeproTech. Erlotinib-sensitive H1650 cells and erlotinib-resistant H1650-M3 cells were kindly provided by Dr. Raffaella Sordella from Cold Spring Harbor Laboratory. Gefitinib-sensitive PC-9 cells and gefitinib-resistant PC-9GR cells were gifted by Prof. Jun Xu and Dr. Ming Liu from Guangzhou Medical University, China. H1975 cells were provided by the American Type Culture Collection. All the cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, HyClone) with Earle’s salts supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine solution (Gibco), 100 U/ml penicillin (HyClone) and 100 μg/ml streptomycin (HyClone) at 37°C, with 5% CO₂, and 90% humidity.

Cell growth, invasion and migration assays

The cytotoxic effects of gefitinib or erlotinib plus metformin were determined by the MTT dye reduction method and BrdU incorporation assay (10). Cell invasion was measured using 24-well 6.5-mm diameter inserts (8.0 μm pore size, Corning Incorporated). The relative cell invasion index was calculated as reported (18). Cell migration was evaluated using single-cell tracking assay as described earlier (19). Cell apoptosis was analyzed by flow cytometry. For more details, please refer to the Supplementary Materials and Methods.
Animal experiments

For xenograft implantation, a total of $2 \times 10^6$ PC-9GR or PC-9 cells were injected subcutaneously into the back next to the left forelimb of 6-week-old female BALB/cA-nu mice (Laboratory Animal Center of Third Military Medical University, Chongqing, China), all of which developed tumors with a size of ~30 mm$^3$ within 5-7 days. The mice were then randomly assigned to four groups (eight mice/group) with or without oral administration of 1mg/ml metformin, or 250 mg/L gefetinib, or both, in drinking water. Tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$ and measured twice a week (Fig. 5A). The animals were kept in individual ventilated cages in compliance with institutional guidelines. All animal protocols were approved by the Ethics Committee of the Third Military Medical University. After 4 weeks, tumor-bearing mice were sacrificed, and tumors were harvested, fixed with 4% paraformaldehyde and embedded in paraffin. To assess survival, the animals were monitored for 90 days until being euthanized. The distribution of survival percentages over time was estimated using the Kaplan-Meier method.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were carried out using the unpaired, two-tailed Student’s t test and statistical significance was assumed at a value of $p < 0.05$. Kaplan-Meier curves were compared using the Log-rank test.

For details of the materials and methods, please refer to the Supplementary Materials and Methods.
RESULTS

Metformin resensitizes EGFR-TKI-resistant human lung cancer cells in vitro

We first performed MTT assays to determine whether metformin could enhance the inhibitory effects of TKIs on the growth of TKI-resistant lung cancer cell lines, PC-9GR and H1650-M3. PC-9 cells were highly sensitive to gefitinib (Fig. 1A), while PC-9GR cells were highly resistant to it (Fig. 1B). Treatment with 5 mM metformin resensitized PC-9GR to gefitinib (Fig. 1B and Supplementary Figure 1). Similarly, H1650-M3 cells, which were resistant to erlotinib treatment, displayed enhanced sensitivity to erlotinib after metformin treatment (Fig. 1C and Supplementary Figure 1). Treatment with 5mM metformin alone for 48 hours slightly decreased viability of PC-9GR cells and H1650-M3 cells (Fig. 1D).

As metformin disrupts mitochondrial respiration, which may affect the results of MTT assay, we then applied BrdU incorporation assay to measure cell proliferation to avoid any non-specific effects of metformin. Our results confirmed that metformin in combination with gefitinib resulted in robust inhibition of cell proliferation in PC-9GR and another well-established resistant cell line, H1975 cells (Fig. 1E and Supplementary Figure 2). To confirm that metformin can be uptaken by the cell lines used in the current study, we next examined the expression of organic cation transporter 1 (OCT1), the transporter required for uptake of metformin, in PC-9, PC-9GR, H1650, and H1650-M3 cells. Results showed that these cell lines expressed high levels of OCT-1, suggesting that metformin can be uptaken by those cell lines (Supplementary Figure 3).

To further determine whether metformin in combination with TKIs has a better inhibitory effect on tumor cell invasion and migration than metformin or TKIs alone, we performed transwell assay and single-cell tracking assay. Both the invasion and motility of resistant PC-9GR cells were increased as
compared with those of parental PC-9 cells. Gefitinib treatment alone ($IC_{25}^{48h}$) had little effect on invasion or motility. Interestingly, metformin alone was able to decrease the invasion ability and migration rate of PC-9GR cells, and could further enhance these effects when combined with gefitinib (Fig. 1F and G). The same finding was observed in H1650-M3 cells treated with metformin, or erlotinib, or both (Supplementary Figure 4). We next analyzed the induction of apoptosis in PC-9GR cells treated with metformin alone or in combination with gefitinib. Flow cytometric analysis revealed that metformin alone enhanced the apoptosis of PC-9GR cells, and the combination therapy further augmented this effect (Fig. 1H). Taken together, these in vitro data suggest that the combined use of metformin and gefitinib resensitizes resistant cells to TKIs and overcomes the acquired TKI resistance in these cells.

Metformin reverses EMT in TKI-resistant lung cancer cells

Knowing that EMT is extensively correlated with therapeutic resistance to EGFR-TKIs (20), we next examined whether metformin could reverse EMT in TKI-resistant cell lines. Typical epithelial morphology and expression of E-cadherin, an epithelial marker, was observed in PC-9 cells and H1650 cells, while mesenchymal morphology and high expression of Vimentin (a marker of mesenchymal phenotype) and SNAIL (a key regulator of EMT) were observed in PC-9GR and H1650-M3 cells. Metformin treatment induced a transition from spindle-like to epithelial-like morphology, as evidenced by the upregulation of E-cadherin and downregulation of Vimentin and SNAIL in both resistant cell lines (Fig. 2, A-D, Supplementary Figure 5). Western blot results further demonstrated that metformin effectively increased E-cadherin expression while suppressed Vimentin and SNAIL expression in the absence or presence of TKIs as indicated (Fig. 2E and Supplementary Figure 6). Based on these findings,
we conclude that metformin reverses EMT in TKI-resistant lung cancer cells.

Metformin decreases IL-6 signaling activation in TKI-resistant lung cancer cell lines

To identify the molecular mechanisms of overcoming acquired TKI resistance by metformin, we next examined the effect of metformin on IL-6 activation in TKI-resistant cell lines, which was reported to be the key mechanism underlying TKI resistance and the promoter of the EMT process (10). We first performed ELISA analysis and real-time PCR analysis, finding higher levels of protein secretion and gene transcription of IL-6 in both resistant cell lines. Metformin treatment significantly decreased IL-6 protein secretion and gene expression in both resistant cell lines (Fig. 2F and Supplementary Figure 7).

STAT3 and AKT, the key components of IL-6 signaling, were highly phosphorylated in both resistant cell lines. Metformin alone effectively downregulated STAT3 and AKT activation in PC-9GR cells and STAT3 activation in H1650-M3 cells, but it had little effect on AKT activation in H1650-M3 cells. Exposure to gefitinib or erlotinib slightly enhanced STAT3 and AKT phosphorylation, whereas metformin in combination with either gefitinib or erlotinib decreased activation of STAT3 and AKT in PC-9GR and STAT3 activation in H1650-M3 cell lines, respectively (Fig. 2E and Supplementary Figure 6). We then investigated the possible mechanism by which metformin reduces STAT3 and AKT activation. Metformin disrupts mitochondrial respiration, leading to an increase in the intracellular AMP:ATP ratio, and resulting in the activation of AMPK by LKB1. Thus we examined activation of AMPK and acetyl coA carboxylase (ACC, a standard indicator of AMPK activity) under metformin treatment. Levels of phosphorylated AMPK and ACC were significantly decreased in resistant cell lines, while metformin alone or in combination with either gefitinib or erlotinib significantly increased the activation of AMPK and ACC (Fig. 2E and Supplementary Figure 6). In addition, all the cell lines used in the
Metformin overcomes IL-6-induced TKI resistance in TKI sensitive lung cancer cells

Given that the stimulation of TKI-sensitive cells by IL-6 could directly decrease erlotinib sensitivity (10), we then investigated whether metformin could overcome IL-6-induced TKI resistance. It was found that 48-h culture in IL-6-containing medium was able to decrease the sensitivity of PC-9 cells to gefitinib. Interestingly, metformin addition restored the sensitivity of PC-9 cells to gefitinib (Fig. 3A). Metformin alone increased sensitivity of PC-9 cells to gefitinib, especially at low level of gefitinib (0.005 μM), although the difference of IC₅₀ value was not significant when compared to the control. Similarly, overexpression of IL-6 in PC-9 cells (named PC-9psb cells) also resulted in decreased sensitivity to gefitinib, while metformin treatment restored gefitinib sensitivity (Supplementary Figure 8). When assessing the effects of IL-6 activation on tumor cell invasion, we found that the addition of IL-6 enhanced invasiveness of PC-9 cells. In contrast, metformin inhibited invasion of PC-9 cells induced by IL-6 (Fig. 3B). Taken together, these results suggest that IL-6 is sufficient to decrease gefitinib cytotoxicity, which can be reverted by metformin.

Next, we examined EMT and IL-6 activation in PC-9 cells treated with IL-6 or IL-6 plus metformin. IL-6 treatment induced EMT in those sensitive cells, as characterized by spindle-shaped morphology, the loss of E-cadherin and the expression of EMT markers Vimentin and SNAIL (Fig. 3C-E). In addition, exposure to IL-6 resulted in significant phosphorylation of its downstream molecules STAT3 and AKT, but inhibited the activation of AMPK and ACC (Fig. 3D and E). Metformin significantly reverted EMT,
diminished phosphorylation of STAT3 and AKT, and enhanced activation of AMPK and ACC (Fig. 3C-E). In summary, our data indicate that the activation of STAT3 and AKT by IL-6 is sufficient to induce gefitinib resistance, acquisition of mesenchymal-like features and invasion ability, while metformin could successfully restore gefitinib sensitivity, reverse EMT and decrease STAT3 and AKT phosphorylation.

_metformin overcomes TKI resistance by inhibiting IL-6 signaling activation_

To establish a causal link between metformin treatment, IL-6 signaling inhibition, and enhanced TKI response, we increased IL-6 signaling in metformin-pretreated TKI-resistant cells by adding IL-6 to the culture medium. Further addition of IL-6 significantly diminished the response of metformin-pretreated PC-9GR cells to gefitinib (Fig. 4A) and the response of metformin-pretreated H1650-M3 cells to erlotinib (Supplementary Figure 9A). In addition, further exposure to IL-6 enhanced the invasiveness of PC-9GR (Fig. 4B) and H1650-M3 cells (Supplementary Figure 9B), decreased the expression of E-cadherin and increased the expression of Vimentin and SNAIL (Fig. 4C-E; Supplementary Figure 9, C-E). IL-6 also restored and enhanced STAT3 and AKT phosphorylation, while inhibited the activation of AMPK and ACC in both cell lines (Fig. 4D and E, Supplementary Figure 9, D and E). Taken together, these findings suggest that metformin overcomes TKI resistance by inhibiting IL-6 signaling activation.

_metformin plus gefitinib potentiates gefitinib-induced anti-tumor activity in PC-9GR/mouse xenografts_

We next tested the possibility that the metformin-based combinatorial therapy is more effective in xenografts established with PC-9GR cells. Treatment with metformin slightly slowed down the tumor
growth in xenografts. Gefitinib alone resulted in xenograft shrinkage, similar to the result obtained by metformin. Importantly, in accord with the above results in cell lines, a synergic effect of metformin in combination with gefitinib was observed upon co-administration of gefitinib and metformin, which further reduced the tumor size in this model (Fig. 5B). After 4 weeks of drug administration when the animals were sacrificed, the combination therapy caused a 58% decrease in tumor volume as compared with the control group (p<0.01), while gefitinib alone and metformin alone both caused a 32% decrease (p<0.05 for both as compared with the control, Fig. 5C). During the experiments, no obvious weight loss was observed in mice treated with metformin, gefitinib, or both (Supplementary Figure 10). Besides, no significant reduction in serum insulin levels or glucose levels was observed with metformin treatment (Supplementary Figure 11). A similar combinatorial effect of metformin and gefitinib was observed in xenografts established with TKI-sensitive PC-9 cells. Gefitinib alone resulted in significant tumor shrinkage in PC-9 xenografts, while metformin in combination with gefitinib further reduced the tumor sizes (p<0.05 as compared with gefitinib alone, Supplementary Figure 12). We then evaluated the survival of PC-9GR xenografts treated with gefitinib, or metformin, or both. Logrank test showed that metformin or gefitinib alone did not prolong the survival significantly as compared with the control (p = 0.07 and 0.14). Treatment with metformin + gefitinib significantly prolonged the survival of mice as compared with either metformin or gefitinib alone (p = 0.044 and 0.043, respectively, Fig. 5D).

Collectively, these results suggest that the combination of metformin with gefitinib may overcome gefitinib resistance in vivo.

The combinatorial effect of metformin with gefitinib in mouse xenografts is associated with EMT reversal and inhibition of IL-6 signaling
To clarify the underlying reason why combination of metformin and gefitinib is more effective than either drug alone, we next analyzed EMT and components of the IL-6 signaling pathway in the context of metformin-based combinatorial therapy in PC-9GR xenografts. Immunofluorescence staining showed high expression of Vimentin and low expression of E-cadherin in the control and gefitinib alone-treated groups, indicating that EMT occurred in both groups. Metformin treatment alone or in combination with gefitinib increased E-cadherin expression and decreased Vimentin expression (Fig. 6A). These results were confirmed by Western blot analysis (Fig. 6D). Similarly, in PC-9 xenografts, metformin in combination with gefitinib increased the expression of E-cadherin and decreased that of Vimentin when compared to the control (Supplementary Figure 13). Next, we examined IL-6 secretion and expression in PC-9GR xenografts. Using ELISA analysis, we detected higher levels of IL-6 in peripheral blood in the control groups. Gefitinib treatment alone did not decrease IL-6 secretion significantly as compared with the control group. IL-6 levels were significantly decreased in both metformin alone-treated and the metformin + gefitinib groups (p<0.05 for both, as compared with the control group, Fig. 6B). These results were confirmed by immunohistochemistry of tumor sections using an anti-IL-6 antibody (Fig. 6C). Finally, Western blot analysis showed that STAT3 and AKT were strongly phosphorylated in the control group and gefitinib treatment increased the phosphorylation of STAT3 and AKT. Metformin alone or in combination with gefitinib inhibited the activation of STAT3 and AKT, but significantly increased the activation of AMPK and ACC (Fig. 6D and Supplementary Figure 14 and 15). The expression of phosphorylated AKT and STAT3 was further confirmed by immunohistochemistry (Supplementary Figure 16). These results suggest that the therapeutic advantage of the combination of metformin with gefitinib is associated with its ability to reverse EMT and inhibit IL-6 signaling in vivo.
DISCUSSION

Human NSCLCs with activating EGFR mutations show an excellent response to treatment with EGFR-TKIs, such as gefitinib and erlotinib. However, nearly all patients succumb to relapse due to drug resistance, for which no effective therapy is available at present. Thus, novel strategies are urgently needed to delay or overcome acquired resistance to EGFR-TKIs. In the current study, we have demonstrated that metformin can effectively overcome drug resistance to erlotinib and gefitinib. Furthermore, metformin plus gefitinib significantly decrease tumor growth in vivo and prolong animal survival.

Clinical implications of metformin plus TKIs to overcome drug resistance

Several epidemiological studies have indicated that metformin uptake can lower the risk of several types of cancer in diabetic patients (21-23). The diabetic patients with breast cancer receiving metformin and neoadjuvant chemotherapy had a higher pathologic complete response rate than did those not receiving metformin (24). In mouse xenografts, metformin exerted comparable effects on tumor regression when it was combined with a 4-fold reduced dose of doxorubicin that is not effective as a monotherapy (14). Metformin inhibited the proliferation of NSCLC (25) and breast cancer cell lines (26), and blocked transformation in an inducible model system (27, 28). These reports, together with our findings that metformin significantly enhances the effect of erlotinib and gefitinib on TKI-resistant cell lines in vitro and in vivo, suggest that metformin has the promising potential to be used as a novel anti-cancer agent.

The dose of metformin we used in in vitro experiments is higher than that used in diabetic patients. However, as mentioned by Iliopoulos et al. (14), metformin accumulates in tissues at the concentration several-fold higher than that in blood, so that a concentration of 1–10 mmol/L might be attained in
tumor tissues during cancer treatment, as discussed elsewhere earlier (17). In previous in vivo experiments testing the anti-tumor effect of metformin on xenografts, the dose of metformin varies from 200 μg/ml (14) to 200 mg/ml (17), and it is diluted in drinking water, present throughout the experiment. In the current study, we used 1 mg/ml metformin, corresponding to 75 mg/kg as reported by Iliopoulos et al (14). The well-established Reagan-Shaw method (29) shows the human equivalent dose (mg/kg) = animal dose (mg/kg) × animal Km/human Km, where Km values are based on body surface area. For a 60 kg human adult, Km is 37, while for a 20 g mouse, it is 3. Thus, the human equivalent of the murine dose of 75 mg/kg is 365 mg in an adult of 60 kg, which is much less than the maximum metformin dose of 2550 mg/day recommended by the Food and Drug Administration. Thus, the in vivo dose of metformin in the current study is within a therapeutic range in humans.

**Mechanistic implications**

Understanding the molecular mechanisms underlying the ability of metformin to overcome TKI resistance is pivotal to develop it as a novel agent to treat NSCLC patients. EGFR TKI treatment resulted in STAT3 activation, which was caused by IL-6 in an autocrine manner (11). Inhibiting IL-6/STAT3 suppressed cancer cell growth and enhanced the sensitivity to anticancer drugs (30). On the other hand, AKT phosphorylation was upregulated in gefitinib-resistant NSCLC cells, and maintenance of PI3K/AKT pathway signaling was associated with therapeutic resistance to EGFR-TKIs. As IL-6 is a major activator of the JAK/STAT3 and PI3K/AKT pathways (31), inhibiting its signaling has emerged as a possible solution to the problem of EGFR-TKI resistance.

In the present study, we observed robust activation of IL-6 signaling pathway in TKI-resistant cells when compared with their parental sensitive cells. In addition, IL-6 overexpression or addition in TKI
sensitive PC-9 cells resulted in gefitinib resistance, accompanied by increased phosphorylation of STAT3 and AKT. Metformin treatment effectively overcomes TKI resistance in established TKI-resistant cell lines, IL-6 stimulated PC-9 cells, and IL-6 overexpressing PC-9psb cells. Furthermore, adding IL-6 into metformin-pretreated TKI resistant cell lines abolished metformin’s effect, restored TKI resistance, and re-activated IL-6 signaling pathway. Thus, our data provide compelling evidence that metformin overcomes acquired resistance to molecular-targeted therapies by inhibiting IL-6 signaling pathways (Supplementary Figure 17).

Besides the inhibitory effect on IL-6 signaling pathway, metformin’s ability to reverse EMT may also play an important role in overcoming TKI resistance. EMT has been associated with resistance to EGFR inhibitor treatment in NSCLCs (20). AXL, an EMT marker, was found to be upregulated in NSCLC patients with acquired resistance to EGFR TKI treatment, and AXL activation is a major cause of EGFR-TKI resistance (32). In the current study, we demonstrated that metformin reversed EMT in TKI-resistant lung cancer cell lines and IL-6 stimulated PC-9 cells. We also found that metformin inhibited IL-6/STAT3 pathway, while IL-6 is capable of inducing EMT in cancer cells (33). Thus, we conclude that EMT reversal by metformin is associated with its ability to overcome TKI resistance.

**Metformin may disrupt the evil axis of TGFβ/IL-6, EMT, cancer stem cells and drug resistance**

Cancer stem cells (CSCs), which constitute a small portion of neoplastic cells, are hypothesized to be critical initiators of cancers and mediate resistance to conventional anti-tumor therapies (34). Lung CSCs have also been successfully isolated from lung cancer cell lines based on SP phenotypes (low Hoechst 33342 staining pattern) (35), and from primary patient tumors (36). These lung CSCs are resistant to most conventional drugs currently used to treat lung cancer patients. On the other hand, lung
cancer cells surviving conventional or targeted therapies exhibited several CSC features, such as strong clonogenic capacity, self-renewal and high tumorigenicity (37, 38). Interestingly, the two inflammation-associated cytokines, IL-6 and transforming growth factor-beta 1 (TGF-β1), are capable of inducing EMT in human breast cancer cells, resulting in generating cells with stem-cell properties (39, 40). Indeed, EMT-CSCs-drug resistance has been proposed to be an emerging axis of evil in the war against cancer (41). In addition, as shown in the current study, TKI-resistant lung cancer cells displayed EMT features and increased activation of IL-6 signaling pathways. Besides, trastuzumab-refractory CSC populations were found to be significantly enriched in the expression of mesenchymal markers, and an increased secretion of TGFβ (42). Thus, TGFβ/IL-6, EMT, CSCs and drug resistance are strongly correlated with each other to maintain the TKI resistance in lung cancer.

In the current study, we found that metformin could inhibit IL-6 secretion, decrease IL-6 signaling activation and reverse EMT. It was also reported that metformin could reverse TGFβ-induced EMT (15). In addition, metformin treatment inhibited the growth of breast CSCs (13), ovarian CSCs (43), pancreatic CSCs (44) and thyroid CSCs (45), possibly by transcriptionally repressing the stem cell property EMT (46). Taken together, metformin emerges as a new therapeutic option to inhibit TGFβ/IL-6 activation, reverse EMT, kill CSCs and then overcome TKI resistance, thus disrupting this evil axis in the war against cancer (Supplementary Figure 18). Given the important role of CSCs in TKI resistance, we are currently examining the effect of metformin on killing CSCs in TKI-resistant lung cancer cells.

**Metformin may alleviate TKI-induced interstitial pneumonia**

Acute interstitial pneumonia is one of the serious adverse effects of TKI treatment (47). IL-6 is known to
induce interstitial pneumonia (48). TKI treatment activates AP-1 in lung cancer cells and promotes IL-6 secretion, which further increases the expression of collagen and α-actin, markers for fibrosis (49). In the current study, we provide sound evidence that metformin can inhibit the IL-6/STAT3 axis. Also, it has been reported that TGF-β is an important inducer of pulmonary fibrosis (50), while metformin can significantly inhibit TGF-β signaling (15). Accordingly, the combination of metformin and EGFR-TKI may be more effective and safer for patients, since this treatment may not only overcome TKI resistance but prevent or alleviate the development of acute interstitial pneumonia.

Therapeutic resistance to EGFR-TKIs is almost inevitable in patients with activating EGFR mutations who initially respond well to therapy. Management of TKI resistance has become the focus of research to lengthen overall survival of these patients. In this study, we have proven for the first time that metformin overcomes TKI resistance \textit{in vitro} and \textit{in vivo} by inhibiting IL-6 signaling pathway and reverting EMT. In addition, we have successfully started a clinical trial (registered in Clinicaltrials.gov: \url{http://www.clinicaltrials.gov/ct2/show/NCT01864681}) to observe the clinical benefits of metformin in combination with gefitinib to treat advanced NSCLC patients with EGFR mutations. Our future research aims at providing more solid evidence to develop metformin in combination with TKIs as a new therapeutic approach to prolonging the survival of NSCLC patients.
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References


Figure Legends

**Figure 1. Metformin overcomes TKI resistance in TKI-resistant human lung cancer cells.** A and B, Metformin increased the sensitivity of gefitinib-resistant cells to gefitinib. Cell viability of parental PC-9 and resistant PC-9GR cells treated with the indicated doses of gefitinib for 48 h were assessed with the MTT method. C, Parental H1650 and H1650-M3 cells were treated with the indicated doses of erlotinib for 48 h. Cell viability, assessed by the MTT method, was expressed as % of control for each time point. D, Treatment with 5 mM metformin alone for 48 h slightly decreased the viability of both PC-9GR and H1650-M3 cells. The data shown represent the mean value of the percentage of viable cells ± SEM (*, p < 0.05 and †, p < 0.01 compared with the control). E, Metformin (5mM) and gefitinib (IC_{25}^{48h}) synergistically inhibited the proliferation of PC-9GR cells, as determined by BrdU incorporation assay. *, p < 0.01 compared with control, †, p < 0.01 compared with metformin alone. Scale bars, 50 μm. F, Metformin (5 mM) and gefitinib (IC_{25}^{48h}) synergistically inhibited the invasiveness of PC-9GR cells. Scale bars: 100 μm. *, p < 0.01 compared with that of PC-9; †, p < 0.05 compared with PC-9GR; ‡, p < 0.05 compared with metformin treatment alone. G, Metformin treatment decreased single cell motility. Each trace represents the movement of a single cell within an hour, with starting point set at (0, 0). H, Metformin in combination with gefitinib enhanced apoptosis of PC-9GR cells. Images are representative of three independent experiments. *, p < 0.01 compared with control; †, p < 0.05 compared with metformin treatment. Met, metformin; Gef, gefitinib; DAPI, 4’, 6-diamidino-2-phenylindole.

**Figure 2. Metformin reverses EMT and decreases IL-6 signaling in TKI-resistant human lung cancer cells.** A and B, Phase-contrast images of parental and resistant cells in the presence or absence of 5 mM metformin for 48 h. Scale bars: 100 μm. C and D, Immunofluorescence staining showed that...
TKI-resistant cell lines expressed high levels of Vimentin and low levels of E-cadherin, which was reversed by metformin treatment. The nucleus were stained with 4’, 6-diamidino-2-phenylindole in the merged images. Scale bars: 30 μm. E, Whole cell protein lysates from different cell lines were immunoblotted with indicated antibodies. Similar results were obtained in three independent experiments. F, Metformin alone or in combination with TKIs significantly decreased IL-6 secretion levels in TKI-resistant H1650-M3 and PC-9GR cells as determined by ELISA assays. *, p < 0.01 compared with parental cell lines, respectively; †, p < 0.01, compared with untreated resistant cell lines, respectively; ‡, p < 0.05, compared with H1650-M3 cells treated with metformin alone. Met, metformin; TKI, tyrosine kinase inhibitor; E-cad, E-cadherin; Vim, Vimentin; Gef, gefitinib; Erl, erlotinib.

Figure 3. Metformin reverses IL-6-induced TKI resistance, EMT and IL-6 signaling activation. A, Metformin reversed IL-6-induced gefitinib-resistance in parental PC-9 cells. Parental PC-9 cells (untreated, or treated with 10ng/ml IL-6 or IL-6 plus 5mM metformin) were incubated with gefitinib at the indicated concentrations. Cell viability was assessed with the MTT method after 48-h treatment. B, Metformin decreased IL-6-enhanced invasiveness of parental PC-9 cells. Scale bars, 100 μm. *, p < 0.01 compared with no treatment group; †, p < 0.01, compared with IL-6 treatment group. C, Metformin reversed IL-6-induced EMT in parental PC-9 cells. Morphology of PC-9 cells with different treatments was shown by phase-contrast images. Expression of E-cadherin and Vimentin was determined by immunofluorescence staining. Cells were counter-stained with 4’, 6-diamidino-2-phenylindole. Scale bars, 100 μm for phase-contrast images and 30 μm for immunofluorescence images. D, Metformin decreased IL-6 signaling activation in IL-6-stimulated parental PC-9 cells. Whole cell protein lysates from PC-9 cells with different treatments were immunoblotted with antibodies as indicated, and β-actin was used to
confirm equal gel loading. Similar results were obtained in three independent experiments. E, Quantification of blots in Fig. 3D. *, p < 0.05 and †, p < 0.01, compared with PC-9 cells, respectively; ‡, p < 0.05 and §, p < 0.01, compared with IL-6 treatment, respectively. Met, metformin; E-cad, E-cadherin; Vim, Vimentin.

Figure 4. Inhibition of IL-6 signaling is essential for metformin to overcome gefitinib resistance in PC-9GR cells. A, Addition of IL-6 increased the viability of metformin-pretreated PC-9GR cells. PC-9GR cells were pretreated with 5 mM metformin for 48 h, and then treated with gefitinib of different doses as indicated, still with 5 mM metformin, or 5 mM metformin plus IL-6 (10 ng/ml). Cell viability was measured 48 hours later. B, IL-6 enhanced the invasion of metformin-pretreated PC-9GR cells. Gefitinib (IC_{50}^{25}) plus metformin (5 mM)-pretreated PC-9GR cells were grown in the presence or absence of IL-6 (10 ng/ml) for 48 h, and then cellular invasiveness was assessed by the transwell assay. Scale bars, 100 μm. *, p < 0.01 compared with that without addition of IL-6. C, Immunostaining showed that addition of IL-6 increased Vimentin expression and decreased E-cadherin expression in metformin-plus gefitinib-pretreated PC-9GR cells. Cells were counter-stained with 4’, 6-diamidino-2-phenylindole. Scale bars, 30μm. D, Addition of IL-6 restored IL-6 signaling activation in metformin-pretreated PC-9GR cells. Whole cell protein lysates from PC-9GR cells with different treatments were immunoblotted with indicated antibodies and β-actin were used to confirm equal gel loading. Similar results were obtained in three independent experiments. E, Quantification of blots in Fig. 4D. *, p < 0.01, compared with that without addition of IL-6, respectively. Met, metformin; Gef, gefitinib; E-cad, E-cadherin; Vim, Vimentin.
Figure 5. Oral administration of metformin together with gefitinib suppresses tumor growth and prolongs survival. A, Schematic figure of the xenograft model used in the current study. B, Tumor volume (mm^3) of PC-9GR cells treated with gefitinib, metformin, and their combination. *, p < 0.05 when compared with the control; †, p < 0.05 as compared with metformin alone; ‡, p < 0.05 as compared with gefitinib alone. C, Macroscopic appearance of the tumors at 4 weeks after drug administration. D, Kaplan Meier survival curves were calculated for the four treatment groups mentioned above. Logrank test demonstrated a statistical difference between the gefitinib plus metformin group and metformin alone, or gefitinib alone groups (p = 0.044 and 0.043, respectively). Metformin or gefitinib alone did not prolong the survival significantly as compared with the control group (p = 0.07 and 0.14, respectively). Met, metformin; Gef, gefitinib.

Figure 6. Metformin in combination with gefitinib reverses EMT and decreases IL-6 signaling in xenograft tumors. A, Metformin plus gefitinib reversed EMT in xenograft tumors. Each panel represents a representative immunostaining of a paraffin-embedded section (4 μm) for E-cadherin in green, Vimentin in red and counter-staining with 4’, 6-diamidino-2-phenylindole (DAPI) in blue. The area indicated by the square is shown at higher magnification. Scale bars, 30 μm. B, Metformin plus gefitinib decreased IL-6 levels in the blood. The chart represents IL-6 levels in the serum of mice from the four groups determined by ELISA. The data are expressed as means ± SEM (n = 8 each). *, p < 0.01 as compared with the control group; †, p < 0.01 as compared with gefitinib group. C, Metformin plus gefitinib decreased IL-6 expression in the tumor tissues. Paraffin-embedded sections (4 μm) from tumor tissues were stained for IL-6 using immunohistochemistry. Scale bars, 80 μm. D, Western blotting analyzed the expression of indicated markers on protein extracts obtained from harvested tumors, and
β-actin was used as a loading control. Met, metformin; Gef, gefitinib.
**Figure 1**

A. Cell viability (% of control) with Gefitinib (μM) for PC-9.

B. Cell viability (% of control) with Gefitinib (μM) for PC-9GR.

C. Cell viability (% of control) with Erlotinib (μM) for H1650-M3.

D. Cell viability (% of control) with Met for PC-9 GR and H1650-M3.

E. Incorporation of BrdU (% of cells) and Relative cell invasion for PC-9GR.

F. Relative cell invasion and Apoptosis (%) for PC-9 and PC-9GR.

G. Annexin V and PI for PC-9 and PC-9GR.

H. Annexin V and PI for PC-9GR with Met, Gef, Gef+Met.
Figure 3

A. Cell viability (% of control) as a function of Gefitinib concentration.

B. Invasion assay showing control, IL-6, Met, IL-6+Met, and Met conditions.

C. Morphology images for IL-6, Met, IL-6+Met, and Met conditions.

D. Western blot analysis showing expression of E-cadherin, Vimentin, SNAIL, P-STAT3, STAT3, P-AKT, AKT, P-AMPK, AMPK, P-ACC, and ACC under different conditions.

E. Bar graph showing fold change of protein levels for PC-9, PC-9+IL-6, and PC-9+IL-6+Met conditions.
Figure 4

A

Cell viability (% of control)

![Graph showing cell viability against Gefitinib (μM)]

PC-9GR+Met
PC-9GR+Met+IL-6

B

Relative cell invasion

![Bar graph showing relative cell invasion with conditions] PC-9GR cells

Gef + + +
Met + + +
IL-6 - + +

C

![Images of E-cadherin, Vimentin, and merged images with conditions]

Gef + + +
Met + + +
IL-6 - + +

D

![Images of E-cadherin, Vimentin, SNAIL, P-STAT3, STAT3, P-AKT, AKT, P-AMPK, AMPK, P-ACC, ACC, and β-actin with conditions]

E

Fold change of protein levels

![Bar chart showing fold change of protein levels with conditions]

PC-9GR+Gef+Met
PC-9GR+Gef+Met+IL-6

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**Figure 5**

A. Schematic diagram of the experimental setup.

- Cells injection: Tumor size ~30 mm³
- Drug application (0w): Water, Met alone, Gef alone, Gef and Met
- Tumor volume measurement
- Sacrifice (4w)

B. Graph showing tumor volume over weeks post-drug application.

- Control
- Met
- Gef
- Gef+Met

C. Images of tumor samples from different treatment groups.

D. Survival rate over days post-drug application.

- Control
- Met
- Gef
- Gef+Met
Figure 6

A

Control

Metformin

Gefitinib

Gef+Met

B

C

D

E-cad/Vim/DAPI  E-cadherin  Vimentin  E-cad/Vim/DAPI  DIC  Merged

Control   Met   Gef   Gef+Met

Production levels (pg/ml)

Control Met Gef Gef+Met

E-cad  Vim  SNAIL  p-STAT3  STAT3  p-AKT  AKT  P-AMPK  AMPK  P-ACC  ACC  β-actin
Metformin sensitizes EGFR-TKI-resistant human lung cancer cells in vitro and in vivo through inhibition of IL-6 signaling and EMT reversal

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