Preclinical Development

Activation of IL-6R/JAK1/STAT3 Signaling Induces De Novo Resistance to Irreversible EGFR Inhibitors in Non–Small Cell Lung Cancer with T790M Resistance Mutation

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Abstract
The secondary T790M mutation in epidermal growth factor receptor (EGFR) is the major mechanism of acquired resistance to EGFR tyrosine kinase inhibitors (TKI) in non–small cell lung cancer (NSCLC). Although irreversible EGFR TKIs, such as afatinib or dacomitinib, have been introduced to overcome the acquired resistance, they showed a limited efficacy in NSCLC with T790M. Herein, we identified the novel de novo resistance mechanism to irreversible EGFR TKIs in H1975 and PC9-GR cells, which are NSCLC cells with EGFR T790M. Afatinib activated interleukin-6 receptor (IL-6R)/JAK1/STAT3 signaling via autocrine IL-6 secretion in both cells. Inhibition of IL-6R/JAK1/STAT3 signaling pathway increased the sensitivity to afatinib. Cancer cells showed stronger STAT3 activation and enhanced resistance to afatinib in the presence of MRC5 lung fibroblasts. Blockade of IL-6R/JAK1 significantly increased the sensitivity to afatinib through inhibition of afatinib-induced STAT3 activation augmented by the interaction with fibroblasts, suggesting a critical role of paracrine IL-6R/JAK1/STAT3 loop between fibroblasts and cancer cells in the development of drug resistance. The enhancement of afatinib sensitivity by inhibition of IL-6R/JAK1/STAT3 signaling was confirmed in in vivo PC9-GR xenograft model. Similar to afatinib, de novo resistance to dacomitinib in H1975 and PC9-GR cells was also mediated by dacomitinib-induced JAK1/STAT3 activation. Taken together, these findings suggest that IL-6R/JAK1/STAT3 signaling can be a potential therapeutic target to enhance the efficacy of irreversible EGFR TKIs in patients with EGFR T790M. Mol Cancer Ther; 11(10): 2254–64. ©2012 AACR.

Introduction
The epidermal growth factor receptor (EGFR) is a proto-oncogene regulating cell proliferation, angiogenesis, and metastasis (1). EGFR abnormalities including amplification, mutation, and overexpression are frequently identified in a variety of tumor types and known to exert a strong oncogenic potential (2).

Gefitinib and erlotinib are first-generation EGFR tyrosine kinase inhibitors (TKI) that block EGFR signaling pathway through reversible binding to EGFR (3). Despite a dramatic initial response to reversible EGFR TKIs in patients with EGFR activating mutations such as exon 19 in-frame deletions or exon 21 L858R point mutation, almost all patients acquire resistance to these agents because of diverse mechanisms. In 50% of these patients, resistance is derived by the occurrence of a secondary T790M mutation in exon 20 of EGFR (4, 5). Therefore, to develop an effective therapy for patients harboring EGFR T790M is important to overcome the acquired resistance to the first-generation EGFR TKIs.

Recently, second-generation EGFR TKIs have been clinically developed. These agents effectively inhibit EGFR activity by irreversible binding to EGFR. Afatinib (BIBW2992) and dacomitinib (PF299804) are representative second-generation irreversible EGFR TKIs currently undergoing clinical trial and have shown promising antitumor activity in NSCLC (6, 7). Because these agents have been reported to exhibit antitumor activity in preclinical
studies using NSCLC cells harboring the EGFR T790M, they are expected to be standard therapeutic options for patients with EGFR T790M (7–9). However, these irreversible EGFR TKIs were more than 100-fold less potent in NSCLC cells with EGFR T790M mutation than in NSCLC cells with EGFR activating mutation (10, 11). Recent clinical study also showed a limited efficacy of afatinib suggesting the necessity of developing a new strategy to improve the efficacy of afatinib (12).

STAT proteins are a family of transcription factors that play a key role in multiple cellular functions (13). Among STAT molecules, STAT3 is known to be constitutively activated in a variety of tumor types such as head and neck, lung, and breast cancer. Persistent STAT3 activation is oncogenic and regulates cell-cycle progression, tumor invasion, metastasis, and angiogenesis (13–16). Several reports showed that inhibition of STAT3 suppresses the growth of cancer cells and enhances the sensitivity to anti-cancer drugs in multiple types of cancers (17–23). Therefore, STAT3 has been considered as a potential target for cancer therapy.

In the present study, we identified that irreversible EGFR TKIs such as afatinib and dacomitinib induce STAT3 activation via autocrine interleukin-6 (IL-6) production and that blockade of IL-6R/JAK1/STAT3 signaling pathway potentiates sensitivity to irreversible EGFR TKIs in NSCLC cells harboring EGFR T790M. These findings suggest that targeting of the IL-6R/JAK1/STAT3 signaling pathway can be an effective therapeutic strategy to enhance the efficacy of irreversible EGFR TKIs for patients with acquired resistance to irreversible EGFR TKIs by acquisition of EGFR T790M.

Materials and Methods

Cell culture

The NCI-H1975 cells (EGFR L858R/T790M) were purchased from the American Type Culture Collection and were not authenticated. The PC9-GR cells (EGFR delE746_A750/T790M) were kindly provided by Lee JC (Korea Institute of Radiological and Medical Science, Seoul, Republic of Korea). Existence of EGFR T790M mutation in PC9-GR cells was identified by direct sequencing. Human fetal fibroblasts MRC5 cells were provided by Cho YS (Asan Medical Center, University of Ulsan, College of Medicine, Seoul, Republic of Korea) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing nonessential amino acids, 10% FBS. All other cells were maintained in RPMI 1640 supplemented with 10% FBS. Cell culture media and supplements were obtained from HyClone.

Reagents and antibodies

Gefitinib, afatinib, and dacomitinib were provided by AstraZeneca, Boehringer Ingelheim Pharma, and Pfizer, respectively. AG490 and P6 were purchased from Calbiochem. Interleukin-6 receptor (IL-6R) neutralizing antibody or IgG1 isotype control was obtained from Santa Cruz Biotechnology. Proliferating cell nuclear antigen (PCNA) antibody was purchased from Dako Cytomation. All other antibodies were purchased from Cell Signaling.

Cell viability assay

Cells were seeded at a density of 1 x 10^4 cells per well into a 96-well culture plate and incubated for 24 hours. After cells were exposed to drugs for 72 hours, 0.5 mg/mL of MTT was added to the medium in the well. After incubation for 4 hours at 37°C, formazan crystals in viable cells were solubilized with 100 uL dimethyl sulfoxide. The optical density of the MTT formazan product was read at 565 nm on a microplate reader. All experiments were conducted in triplicate. MTT was purchased from Amresco.

Western blot analysis

Cell lysates were prepared as previously described (24). Equal amounts of protein were fractionated by SDS-PAGE and then transferred onto a nitrocellulose membrane (BioRad). After blocking with 5% skim milk, the membrane was incubated with the primary antibodies at 4°C overnight. Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and ECL solution (Amersham-Pharmacia Biotech).

siRNA transfection

siRNA transfection was conducted with Lipofectamine RNAiMAX reagent according to the manufacturer’s instructions (Invitrogen). Briefly, the cells were transfected with 30 umol/L of siRNA for 6 hours with Lipofectamine RNAiMAX reagent and replaced with fresh growth medium. The following day, cells were treated with afatinib or dacomitinib for MTT assay or Western blot analysis.

IL-6 quantification

Supernatants from cultured cells were collected and centrifuged for 5 minutes at 6,000 g to remove dead cells and cellular debris. Following centrifugation, the supernatants were stored at −80°C. The culture cells were harvested by trypsinization and counted for normalization. ELISA for IL-6 in supernatants was conducted in triplicate using ELISA kits according to the manufacturer’s instructions (R&D Systems).

Quantitative real-time PCR

One microgram of total RNA was reverse-transcribed using SuperScript III RT (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time PCR was conducted on a 7500 Real-Time PCR System (Applied Biosystems) using the SYBR Green detection protocol. For each sample, the mRNA level of IL-6 was normalized against 18S rRNA and the ratio of normalized mRNA to the control was determined using the comparative Ct method. The primers used for real-time PCR are as follows: IL-6, (F) 5’TCTCCACAAGCGCCTTCG-3’, (R) 5’TCTCCACAAGCGCCTTCG-3’.
5′-CTCAGGGCTGAGTCCG-3′; 18S rRNA, (F) 5′-TTCCGAACCTGAGGCCCATGAT-3′; and (R) 5′-TTTCGCTC-TGGTCC-GTCTTG-3′.

Preparation of fibroblast-conditioned medium
MRC5 fibroblasts were seeded at 1.5 × 10⁶ cells in 100-mm culture dishes. The following day, cells were washed twice with serum-free media and changed to media containing 0.5% serum. After 48 hours incubation, the conditioned media were collected by centrifugation for 5 minutes at 3,000 rpm. Aliquots of conditioned media were stored at −80°C. It was diluted 1:2 with fresh complete cell culture media for further experiments.

Xenograft studies
All experiments were in accordance with the Yonsei University Medical College guidelines and regulations approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Six-week-old nude mice were injected subcutaneously with PC9-GR cells (5 × 10⁶). When tumor volumes reached approximately 50 mm³, mice were randomly allocated into groups of 6 animals to receive either vehicle control, afatinib alone, P6 alone or afatinib and P6 together. Afatinib was suspended in 0.5% (w/v) methylcellulose containing 0.4% Tween 80 and administered once daily by oral gavage (5 mg/kg). P6 was dissolved in saline and administered by intraperitoneal injection at a daily dosage of 10 mg/kg. Tumor size was measured every 2 days using calipers. The average tumor volume in each group was expressed as mm³ and calculated according to the equation for a prolate spheroid: tumor volume = 0.523 × (large diameter) × (small diameter)².

Immunohistochemistry
Sacrificed tumors (n = 3 per group) were fixed in 10% neutral buffered formalin for 24 hours, embedded in paraffin and sectioned (4 μm). Tumor tissue sections were deparaffinized, soaked in ethanol, and incubated in 3% H2O2 for 10 minutes at 3,000 rpm. Aliquots of conditioned media were stored at −80°C. It was diluted 1:2 with fresh complete cell culture media for further experiments.

Results
STAT3 activation upon afatinib treatment mediates de novo resistance to afatinib in NSCLC cells harboring an EGFR T790M mutation.

Although both H1975 and PC9-GR cells showed relative sensitivity to afatinib (Fig. 1A) compared with gefitinib, the IC50 of afatinib for inhibition of cell viability was more than 100 nmol/L (Fig. 1B). As shown in Supplementary Fig. 1, treatment of afatinib for 24 hours induced cell-cycle arrest at G0–G1 phase rather than cell death. At the same time point, HER family, the target of afatinib, was dramatically inactivated even at 10 nmol/L of afatinib (Fig. 1C). These data indicate the existence of HER family-independent de novo resistance mechanism to afatinib. To find out de novo resistance mechanisms of afatinib in those cells, we examined the alterations of 3 main downstream effectors in EGFR signaling, AKT, extracellular signal-regulated kinase (ERK), and STAT3, upon afatinib treatment. Whereas AKT and ERK were inactivated in a dose-dependent manner by afatinib, STAT3 was surprisingly hyperactivated by afatinib in both cells (Fig. 1D). We tested if afatinib-induced STAT3 activation mediates de novo resistance to afatinib. In both H1975 and PC9-GR cells, STAT3 knockdown using siRNA increased the antiproliferative effects of 100 nmol/L afatinib from 7.9% to 62.9% (P < 0.001) and from 16.1% to 48.2% (P < 0.001), respectively (Fig. 1E). Antiproliferative effects of siSTAT3 alone were detected only in H1975 cells.

Next, we confirmed the increased sensitivity to afatinib through STAT3 knockdown by detecting the cleaved caspase-3, an apoptotic marker. Consistently, the cleaved caspase-3 was detected by STAT3 knockdown alone in H1975, but not in PC9-GR, and afatinib-induced caspase-3 cleavage was markedly increased by STAT3 knockdown in both cells (Supplementary Fig. S2). These findings suggest that activation of STAT3 is involved in de novo resistance to afatinib and that inhibition of STAT3 increases sensitivity to afatinib in NSCLC cells with EGFR T790M.

Afatinib-induced STAT3 activation is JAK1 dependent.
To identify the specific upstream regulator of afatinib-induced STAT3 activation, we detected the activation of Janus-activated kinase (JAK) family, which are the main upstream regulators of STAT3 (25). In the presence of 100 nmol/L afatinib, JAK1 and JAK2 were activated in both H1975 and PC9-GR cells but TYK2 was activated only in H1975 cells (Supplementary Fig. 3). As shown in Fig. 2A, knockdown of JAK1, but not JAK2 or TYK2 abrogated the afatinib-induced STAT3 activation in H1975 cells. Similarly, afatinib-induced STAT3 activation in PC9-GR cells was only blocked by silencing of JAK1 but not JAK2. In PC9-GR cells, TYK2 knockdown was not conducted because TYK2 was not activated upon afatinib treatment. To confirm the JAK1-dependent STAT3 activation, we tested if STAT3 is inhibited by treatment of pyridone 6 (P6), pan-JAK inhibitor, or AG490, a JAK2-specific
inhibitor. In both H1975 and PC9-GR cells, only P6 treatment, but not AG490 treatment, blocked the afatinib-induced STAT3 activation (Fig. 2B).

Next, we examined whether JAK1 inhibition can overcome de novo resistance to afatinib in both cells. As a result of MTT assay, the antiproliferative effect of afatinib in both cells was significantly increased by treatment of JAK1-specific siRNA or P6 as shown in Fig. 2C and D. As shown in Supplementary Fig. 4, JAK2 knockdown did not affect the sensitivity to afatinib in both cells. Taken together, these results suggest that afatinib-induced STAT3 activation is specifically mediated by JAK1 in NSCLC cells with EGFR T790M.

Afatinib activates IL-6R signaling pathway via autocrine IL-6 production in EGFR T790M harboring cells

It is well known that STAT3 can be activated by growth factor receptors such as EGFR and c-MET or nonreceptor tyrosine kinase such as c-Src (26). Herein, EGFR (Fig. 1B) and c-MET (Supplementary Fig. 5) were completely inhibited by afatinib. Also, afatinib-induced STAT3 activation was not blocked by treatment of PP2, a c-Src inhibitor (Supplementary Fig. 6). Collectively, these results indicate that EGFR, c-MET, and c-Src are not involved in the afatinib-induced STAT3 activation.

Recently, it has been reported that STAT3 activation via IL-6R plays a role in multidrug resistance in cancer cells (27–29). Furthermore, several reports showed that autocrine IL-6 production is implicated in resistance to anticancer drugs (30–33). On the basis of these reports, we first examined if blockade of IL-6R inhibits afatinib-induced STAT3 activation. In both H1975 and PC9-GR cells, treatment of IL-6R neutralizing antibody or IL-6R-specific siRNA completely suppressed afatinib-induced STAT3 activation and significantly increased the antiproliferative effect of afatinib (Fig. 3A–D). Therefore, these data suggest that afatinib-induced STAT3 activation occurs via IL-6R in H1975 and PC9-GR cells.

Next, we tested if afatinib changes IL-6 production in both cells. As shown in Fig. 3E, IL-6 mRNA levels were elevated about 2-fold upon afatinib treatment in both cells.
Consistently, as a result of ELISA assay, at 24 hours after afatinib treatment, IL-6 secretion was significantly elevated by 1.7-fold compared with control in H1975 cells. Also, in PC9-GR cells, IL-6 secretion was markedly increased by 1.3-fold and 1.9-fold at 12 and 24 hours of treatment of afatinib, respectively, compared with control cells (Fig. 3F). Taken together, these data show that NSCLC cells with EGFR T790M show low sensitivity to afatinib by activation of an IL-6R signaling pathway via autocrine IL-6 production. NSCLC cells harboring EGFR T790M show a strong resistance to afatinib through hyperactivation of an IL-6R signaling pathway in the presence of tumor-stromal fibroblast interactions

Because IL-6 is predominantly derived from fibroblasts in vivo (34), we hypothesized that cross-talk to fibroblasts would reduce the susceptibility to afatinib through activation of the IL-6R/JAK1/STAT3 signaling pathway in cancer cells. To elucidate the potential role of the interaction between fibroblasts and cancer cells in the susceptibility to afatinib, we tested the sensitivity to afatinib in the absence or presence of conditioned medium from MRC5 lung fibroblasts (MRC5-CM). As shown in Fig. 4A, MRC5 cells were shown to secrete IL-6. Both H1975 and PC9-GR cells were more resistant to afatinib in the presence of MRC5-CM than in the absence of MRC5-CM, and P6 treatment significantly increased the susceptibility to afatinib in the absence as well as in the presence of MRC5-CM (Fig. 4B). Consistent results were obtained from transwell coculture experiments between cancer cells and MRC5 fibroblasts (Fig. 4C). As shown in Fig. 4D and E, afatinib-induced STAT3 activation was enhanced in the presence of MRC5-CM and inhibited by treatment of P6 or IL-6R neutralizing antibody in the absence as well as in the presence of MRC5-CM. However, the treatment of MRC5-CM did not affect the inactivation of AKT and ERK by afatinib in both cells. These findings indicate that interaction with fibroblasts potentiates de novo resistance of NSCLC cells with EGFR T790M mutation to afatinib through activation of the IL-6R/JAK1/STAT3 signaling pathway in cancer cells.

The activation of IL-6R/JAK1/STAT3 signaling may be a common mechanism of de novo resistance to irreversible EGFR TKIs in NSCLC cells with EGFR T790M.

Although dacomitinib (Fig. 5A) had the promising inhibitory effects on EGFR and HER2 in the low
In the nanomolar range in H1975 and PC9-GR cells, it showed limited efficacy in the inhibition of cell viability with IC₅₀ values of around 1 micromole in both cells (Supplementary Fig. S7). We tested if STAT3 activation is also involved in de novo resistance to dacomitinib in both cells. As shown in Fig. 5B, treatment of dacomitinib activates STAT3 but inactivates AKT and ERK in both cells. Similar to afatinib, dacomitinib-induced STAT3 activation was blocked by treatment of P6 of JAK1-specific siRNA in both cells (Fig. 5B and C). In MTT assay, sensitivity to dacomitinib was increased by combination treatment of P6 in both cells (Fig. 5D). Therefore, these data indicate that the low efficacy of dacomitinib is driven by STAT3 activation and the involvement of JAK1/STAT3 activation might be a general phenomenon in de novo resistance to the irreversible EGFR TKIs in NSCLC cells with EGFR T790M mutation.

**Inhibition of the JAK/STAT3 signaling potentiates the antitumor activity of afatinib in PC9-GR xenograft models**

To further examine whether inhibition of the JAK/STAT3 signaling pathway enhances the antitumor activity of afatinib in vivo, we tested the effects of P6, afatinib, and their combination on the growth of PC9-GR xenograft tumors established in nude mice. At the end of the...
xenograft study, the combined treatment of P6 and afatinib showed a strong inhibition of tumor growth compared with controls (\( P < 0.001 \)) or to treatment of P6 (\( P < 0.01 \)) or afatinib alone (\( P < 0.01 \); Fig. 6A). The synergistic antitumor effect of the combination with P6 and afatinib was also detected by immunohistochemistry (IHC) staining for PCNA, a marker for cell proliferation. Compared with controls or treatment with P6 or afatinib alone, the combination treatment with both agents showed a strong reduction in PCNA staining (Fig. 6B). Consistent with in vitro observations, IHC results showed that the accumulation of active STAT3 in the nucleus was induced by the treatment of afatinib and markedly blocked by treatment of P6. The staining for active AKT or ERK was decreased by the treatment of afatinib with or without P6 (Fig. 6B).

**Discussion**

In this study, we found that activation of the IL-6R/JAK1/STAT3 signaling pathway by afatinib mediates de novo resistance to afatinib in NSCLC cells harboring EGFR T790M. Our results showed that IL-6 secreted from lung fibroblasts potentiated primary resistance to afatinib in cancer cells via hyperactivation of STAT3 in a paracrine manner. In addition, we identified the role of the IL-6R/JAK1/STAT3 signaling pathway in the hyposensitivity to...
dacomitinib, suggesting that the activation of IL-6R/JAK1/STAT3 loop can be a common de novo resistance mechanism to irreversible EGFR TKIs.

Currently, no approved therapy exists for advanced NSCLC patients who fail reversible EGFR TKIs. Although afatinib has been expected to overcome EGFR T790M-mediated acquired resistance to first-generation reversible EGFR TKIs, a recent phase III study of afatinib failed to show overall survival improvement in gefitinib or erlotinib resistant patients, with response rate of less than 10% (12). The study population comprised a highly selective population that was sensitive to previous EGFR TKIs, suggesting a significant proportion of the enrolled patients had the EGFR activating mutation. Given that the acquisition of EGFR T790M is the main acquired resistance mechanism to reversible EGFR TKIs in those patients, a significant proportion of the patients enrolled in the study might have the T790M mutation. Therefore, contrary to reversible EGFR TKIs, the deciphering of de novo resistance mechanisms rather than acquired resistance mechanisms has become a top research priority in irreversible EGFR TKIs. Although development of therapy to overcome de novo resistance to afatinib is required in the clinical setting, there are no published studies to date on de novo resistance mechanism to afatinib. To our knowledge, we firstly showed a role of IL-6R/JAK1/STAT3 signaling pathway in de novo resistance to afatinib in NSCLC cells with EGFR T790M.

Recent studies showed that NSCLC cells harboring EGFR T790M are also at least 10 times more resistant to dacomitinib than NSCLC cells with EGFR sensitizing mutation, indicating the existence of de novo resistance mechanism to dacomitinib (9, 35). In our results, dacomitinib as well as afatinib also induced JAK1-dependent STAT3 activation and its antitumor activity was enhanced through inhibition of the JAK1/STAT3 signaling in NSCLC cells with EGFR T790M. Although further study is needed, these data suggest that STAT3 activation induced by drug treatment is the common de novo resistance mechanism to irreversible EGFR TKIs in at least NSCLC cells harboring EGFR T790M.

Herein, among the main downstream effector molecules of EGFR, AKT, and ERK were dramatically inactivated upon afatinib treatment, whereas STAT3 was paradoxically hyperactivated via increase of autocrine IL-6 production. We did not show how autocrine IL-6 production is increased upon afatinib treatment. Given that STAT3 is known as a transcription factor for IL-6 transcription as well as a downstream effector molecule of IL-6

Figure 5. Activation of IL-6R/JAK1/STAT3 signaling pathway mediates de novo resistance to dacomitinib. A, chemical structure of dacomitinib. B, cells were treated with the indicated doses of dacomitinib and/or 1 μmol/L P6 for 24 hours. C, after 24 hours posttransfection of siRNAs, cells were incubated with 100 nmol/L dacomitinib for 24 hours. D, cells were treated with the indicated doses of dacomitinib and/or P6. After incubation for 72 hours, MTT assay was conducted.
autocrine IL-6 production by afatinib can be explained by the activation of a positive feedback loop for IL-6-STAT3 axis. Activation of NF-κB is another possible explanation for the autocrine IL-6 production by afatinib. IL-6 is well known as a key downstream target of NF-κB (37). Recently, it was reported that resistance to docetaxel is mediated by increased IL-6 production via NF-κB activation in prostate cancer (38). In addition, it was also reported that autocrine IL-6 production correlates with NF-κB activation in chronic lymphocytic leukemia (CCL) and high IL-6 level is associated with poor prognosis in CCL patients (39). These reports support our hypothesis that NF-κB activation is involved in autocrine IL-6 production upon afatinib treatment.

Also, we showed that the paracrine action of IL-6 secreted by fibroblasts potentiates the IL-6R signaling pathway, resulting in an increase of resistance to afatinib in cancer cells. Tumor microenvironment is known to protect cancer from anticancer therapy–induced apoptosis through secretion of soluble factors such as cytokines and growth factors (40). Among soluble factors secreted from stromal cells in tumor microenvironment, IL-6 is the most widely studied factor and is known to induce resistance to anti-cancer drugs in different types of cancer (28, 29, 31). Because the main sources of IL-6 such as fibroblasts, macrophages, or endothelial cells are enriched in tumor regions (34, 41), it is possible that IL-6 levels are high in the tumor microenvironment and responses to
afatinib in tumors are reduced by IL-6 in the tumor microenvironment. Indeed, it was reported that IL-6 is detected at higher levels in tumor-associated stroma than in normal bone marrow stroma (42). Furthermore, several studies showed that serum IL-6 levels are elevated in patients with lung cancer than in normal individuals (43, 44). Therefore, the combination therapy of an IL-6R/JAK1/STAT3 pathway inhibitor and afatinib or dacomitinib may be an effective therapeutic option in the clinical setting.

Interestingly, as seen in this study, although PC9-GR cells, but not H1975 cells, have weak basal STAT3 activity, both cells showed the primary resistance to afatinib through induction of STAT3 activation upon afatinib treatment. Whereas the cytotoxic effect by inhibition of the IL-6R/JAK1/STAT3 signaling pathway alone was detected only in H1975 cells, the synergistic antitumor effects of the inhibition of the IL-6R/JAK1/STAT3 signaling pathway and afatinib were detected in both cells. Although several studies suggested that basal STAT3 activity is a potential predictive marker for the efficacy of anti-cancer drugs (22, 45–47), our findings suggest that basal STAT3 activity is not sufficient as a predictive marker for the efficacy of afatinib alone or combination therapy with STAT3 inhibition and afatinib.

In conclusion, we showed that irreversible EGFR TKIs such as afatinib and dacomitinib induce STAT3 activation via IL-6R/JAK1-dependent manner and the inhibition of STAT3 activation is effective to overcome de novo resistance to irreversible EGFR TKIs in NSCLC cells harboring EGFR T790M. These data suggest that new strategies targeting IL-6R/JAK1/STAT3 can be a potential therapeutic option to enhance sensitivity to irreversible EGFR TKIs in patients with the secondary EGFR T790M. Several agents targeting the IL-6R/JAK/STAT3 signaling pathway such as siltuximab (IL-6 neutralizing monoclonal antibody; ref. 48), OPB-31121 (STAT3 decoy oligonucleotides; ref. 49), or AZD1480 (a small molecule inhibitor for JAK; ref. 50), which have been clinically developed, may be suitable candidates for future combined therapy with irreversible EGFR TKIs.

Disclosure of Potential Conflicts of Interest
J.G. Christensen is an employee and shareholder of Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: S.M. Kim, J.H. Kim, B.C. Cho
Development of methodology: S.M. Kim, B.C. Cho
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Kim, O.-J. Kwon, B.C. Cho
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