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PRECLINICAL STUDIES

FGFR leads to sustained activation of STAT3 to mediate resistance to EGFR-TKIs treatment

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Summary

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have led to great advances in the treatment of non-small cell lung cancer (NSCLC), but the emergence of drug resistance severely limits their clinical use. Thus, elucidation of the mechanism underlying resistance to EGFR-TKIs is of great importance. In our study, sustained activation of STAT3 was confirmed to be involved in resistance to EGFR-TKIs, and this resistance occurred regardless of exposure time, EGFR-TKIs type, and even cancer cell type. Mechanistically, the sustained activation of STAT3 was not related to gp130/JAK signalling pathway or HER2/EGFR heterodimer formation, while related to the expression and activation levels of STAT3. Furthermore, FGFR was shown to bind more strongly to STAT3 after gefitinib treatment, and the inhibition of FGFR reduced the phosphorylation of STAT3, thereby counteracting the effects of EGFR-TKIs and resulting in the synergistic inhibition of cancer cell proliferation. Taken together, the FGFR/STAT3 axis mediates the sustained activation of STAT3 upon EGFR-TKI treatment. This finding elucidates a new mechanism underlying drug resistance to EGFR-TKIs that the FGFR/STAT3 axis mediates the sustained activation of STAT3, providing theoretical support for considering the combination of TKIs and FGFR inhibitors in clinical cancer treatment.

Keywords STAT3 · EGFR-TKIs · Drug resistance · FGFR

Introduction

It is well known that lung cancer is one of the most common types of cancer, and non-small cell lung cancer (NSCLC) accounts for almost 85% of all cases of lung cancer [1]. Although great efforts have been made to develop single-target drugs that are specific for the driver genes of NSCLC, resistance is still inevitable.

Gefitinib, a first-generation reversible epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), has been approved for the treatment of advanced or metastatic NSCLC [1, 2]. The first-generation EGFR-TKIs, including gefitinib and erlotinib, are found to be most effective in patients with activating mutations in EGFR, however, almost all NSCLC patients who initially respond well to therapy ultimately develop acquired resistance [3]. Substantial efforts have been made to elucidate the mechanisms underlying acquired resistance to EGFR-TKIs, including EGFR-T790M mutation, HER2 amplification, MET amplification, etc. [4]. Additionally, a small number of NSCLC patients who harbour EGFR exon 20 insertion mutations, K-Ras mutations, B-RAF mutations, or sustained STAT3 activation are initially insensitive to gefitinib due to primary resistance. Even though increasing literatures have focused on the resistance to TKIs, the underlying mechanism remains to be elucidated.

STAT3 is a key signalling molecule of the JAK/STAT3 signalling pathway downstream EGFR. The sustained activation of STAT3 promotes the upregulation of cyclic regulatory

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factors and anti-apoptosis factors in tumour cells as well as the abnormal proliferation and worsening of tumours. Wu K et al. [5] showed that sustained activation of STAT3 was an intrinsic mechanism of resistance to EGFR-TKIs and that the combined inhibition of EGFR and STAT3 enhanced the efficacy of gefitinib or other EGFR-TKIs in the treatment of lung cancer. STAT3 activation is triggered by multiple signalling pathways. However, the mechanism underlying the sustained activation of STAT3, as a downstream molecule of EGFR, in the presence of EGFR-TKIs remains unclear; although many reports have appeared, there are always contradictions and inconsistencies among the findings.

In our paper, we demonstrate that the FGFR/STAT3 axis, rather than the gp130/JAK pathway or EGFR/HER2 heterodimers, plays an important role in the sustained activation of STAT3 upon EGFR-TKIs treatment. Moreover, the combination of EGFR-TKIs and FGFR inhibitors can enhance cell sensitivity to EGFR-TKIs; this finding provides a theoretical basis for the use of combinatorial TKI and FGFR inhibitor therapy in NSCLC treatment.

Materials and methods

Kinase inhibitors

Gefitinib was synthesized by the School of Medicine and Pharmacy, Ocean University of China. The purity of the compound was determined by HPLC (Agilent 1260 HPLC system, Agilent Technologies, CA, USA), and the data indicated that the compound had a purity greater than 99%. Afatinib was purchased from J&K Scientific Ltd. Erlotinib and stattic were obtained from Sigma-Aldrich. PD173074 was obtained from Selleck Chemicals. All the compounds were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM or 20 mM and stored at -20 until use in *in vitro* studies.

Cell culture

NCI-H1975 cells were cultured in RPMI-1640 (Life Technologies). A549 cells were grown in F12K (Life Technologies). The HCT116, MCF-7ADM (resistance to Adriamycin), 293T and NIH-3T3 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies). All of the cell lines were obtained from American Type Culture Collection (ATCC). All the cells were maintained in medium supplemented with 10% FBS (Gibco), 2 mM L-glutamine, and 1% penicillin-streptomycin and propagated as monolayer cultures at 37 °C in a humidified 5% CO_2 incubator.

Cell viability assay

A modified tetrazolium salt assay was used to measure the inhibition of cancer cell growth. Cancer cells were plated in a 96-well microtiter plate containing 0.09 ml of growth media per well and incubated for 24 hours. Then, the cells were treated with TKIs at the indicated concentrations for 72 hours. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well of the 96-well plate. After 4 hours of incubation, the formazan product was dissolved and spectrophotometrically quantified at a wavelength of 570 nm. All the experimental data were obtained from six replicates, and all the experiments were repeated at least four times. The inhibition rate of each sample was calculated based on the A570 values as follows: % inhibition rate = (A570 nm control-treated cells /A570 nm control × 100%). The IC_{50} was defined as the concentration that led to 50% inhibition of cell viability.

Immunoblotting assay and antibodies

Cells were incubated with TKIs at the indicated concentrations and for the indicated durations, washed with twice PBS, lysed on ice for 30 minutes in loading buffer and then boiled for approximately 10 minutes. The protein concentration was determine with a BCA reagent. Equal amounts of protein were electrophoresed in 6–10% SDS-PAGE gels, transferred to nitrocellulose membranes (Pall), probed with antibodies and detected by chemiluminescence (Pierce).

The antibodies to detect EGFR, phosphor-EGFR (Tyr 1068), STAT3, phosphor-STAT3 (Tyr 705), JAK1, phosphor-JAK1 (Tyr 1034/1035), JAK2, phosphor-JAK2 (Tyr 1007/1008), phosphor-Akt (Ser 473), β-Actin and Tubulin were obtained from Cell Signaling Technology. The antibodies to detect HER2, gp130 and p-gp130 were purchased from Santa Cruz Biotechnology. All the primary antibodies were diluted 1:1000. Anti-rabbit and anti-mouse (1:5000) HRP-conjugated antibodies were used as the secondary antibodies.

Co-immunoprecipitation (CO-IP)

For co-immunoprecipitation, 200 to 500 mg of protein lysate was incubated with antibody overnight at 4 °C. The next day, 40 μl of protein A agarose beads were added and incubated for 2–4 hours at 4 °C, and then, 700 μl of a wash solution (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 7.5) containing PMSF was added. The mixture was centrifuged at 2500 rpm for 3 minutes at 4 °C. The steps described above were repeated 5–6 times. Then, 40–60 μl of 2 × loading buffer was added, and the sample was boiled for 5–10 minutes and centrifuged. Then, the supernatant was transferred to a

new 1.5 ml EP tube and stored at -20 °C until Western blot analysis.

Retroviral plasmid propagation and infections

The following retroviral vectors were used: EGFR-L858R/T790M (#32,073), EGFR-WT (#11,011), EGFR-L858R (#11,012), EGFR-ASVins (#32,066), EGFR-insH (#32,067), and puro-HER2 (#40,978). All of these vectors were obtained from Addgene. EGFR D770-N771 insNPG, gag/pol and VSV-G were kind gifts from Dr. Heidi Greulich at Harvard Medical School. The specific experimental procedures were previously described [3].

siRNA transfection

siRNA transfection was conducted with the Lipofectamine® 3000 reagent according to the manufacturer's instructions (Invitrogen). Briefly, the cells were transfected with a final concentration of 20 nmol/L siRNA for 4–6 hours with the Lipofectamine® 3000 reagent, and then, the medium was replaced with fresh growth medium. After transfection for 48 hours, the cells were treated with the compounds or DMSO for 0.5 hour, and cell lysate protein samples were used for Western blot analysis. The siRNA gene sequences were as follows: JAK1 siRNA: 5'-CCUGGCCAUCUCACACUAUTT-3', JAK2 siRNA: 5'-CCUGGUGAAAGUCCCAUAUTT-3', gp130 siRNA: 5'-GGCAUACCUUAAACAAGCUTT-3', and STAT3 siRNA: 5'-CCACUUUGGUGUUUCAUAATT-3'. All of these siRNA molecules were obtained from Shanghai GenePharma Co., Ltd.

Chemical crosslinking of EGFR-WT/HER2 in transfected NIH-3T3 cells

Cells were seeded in 6-well plates at 2.0×10^5 cells per well, and the following day, the cells were transfected with gp130 siRNA for 48 hours. Then, the cells were washed twice with cold PBS and incubated with 1.0 mM BS^3 (Thermo Scientific) for 1 hour at room temperature. The reactions were quenched by incubation with 20 mM Tris (pH 7.4) for 15 minutes at room temperature. The cells were then lysed with 2 × loading buffer and immunoblotted to detect the indicated proteins.

Synergism and CI analyses

H1975 cells were seeded in quadruplicate in 96-well plates and treated with gefitinib or PD173074 alone or with the combination of gefitinib and PD173074 at the indicated doses. MTT assays were performed after 72 hours of treatment. The synergistic effectiveness of the inhibitors was analysed by using CompuSyn software. CI < 1 indicates synergistic effects, CI > 1 indicates antagonistic effects, and CI = 1 indicates additive effects between the two drugs. The methodology behind the synergistic effect analysis were previously described [6].

Statistical analysis

The data are represented as the mean ± SD. $P < 0.05$ was considered statistically significant according to the *t* test.

Results

Activation of STAT3 mediates resistance to EGFR-TKIs in NSCLC cells

Both gefitinib and erlotinib are first-generation EGFR-TKIs, while afatinib, a second-generation EGFR-TKI, is capable of inhibiting both EGFR and HER2. It has been reported that resistance to these three compounds is related to the activation of STAT3 [7]. First, we determined the effects of these three compounds on the viability of H1975 cells using the MTT assay. As shown in Fig. 1a, the IC_{50} values of gefitinib, erlotinib and afatinib after treatment of H1975 cells for 72 hours were 12.12 μM, 9.44 μM, and 0.45 μM, respectively. The IC_{50} value of gefitinib after treatment of another lung cancer cell line, A549, was 9.32 μM, and the IC_{50} value of gefitinib after treatment of HCT116 colon cancer cells and MCF-7ADM breast cancer cells was approximately 26 μM (data not shown). Results showed that gefitinib was resistant in all cells tested, erlotinib was moderately resistant in H1975 cells, and afatinib was more sensitive in H1975 cells.

According to previous literature reports [5, 7–9], EGFR activation was significantly inhibited after 24 hours of gefitinib exposure, but downstream STAT3 activation was not inhibited in H1975 cells and HCC827 cells harbouring mutant EGFR or in A549 cells harbouring wild-type EGFR. Consistent with the previously reported results, p-EGFR was potently inhibited in H1975 cells after 24 hours of gefitinib treatment in a dose-dependent manner, however, downstream p-STAT3 was continuously activated (Fig. 1b). Similarly, in A549 cells (Fig. 1c), downstream p-STAT3 was not inhibited. We then determined the combined effect of gefitinib and the STAT3 inhibitor stattic on H1975 cell viability. As shown in Fig. 1d, the combination of stattic and gefitinib synergistically inhibited the proliferation of H1975 cells with CI value < 1, indicating STAT3 activation was responsible for gefitinib resistance. In response to long-term gefitinib exposure, the activation of STAT3 could be affected by multiple cytokines in cell culture supernatants through feedback pathways, to explore the mechanism underlying sustained activation of STAT3, the effect of gefitinib on STAT3 activation after short-term (0.5 hour) treatment was examined. As shown in Fig. 1e and f, after gefitinib treatment for 0.5 hour, p-EGFR

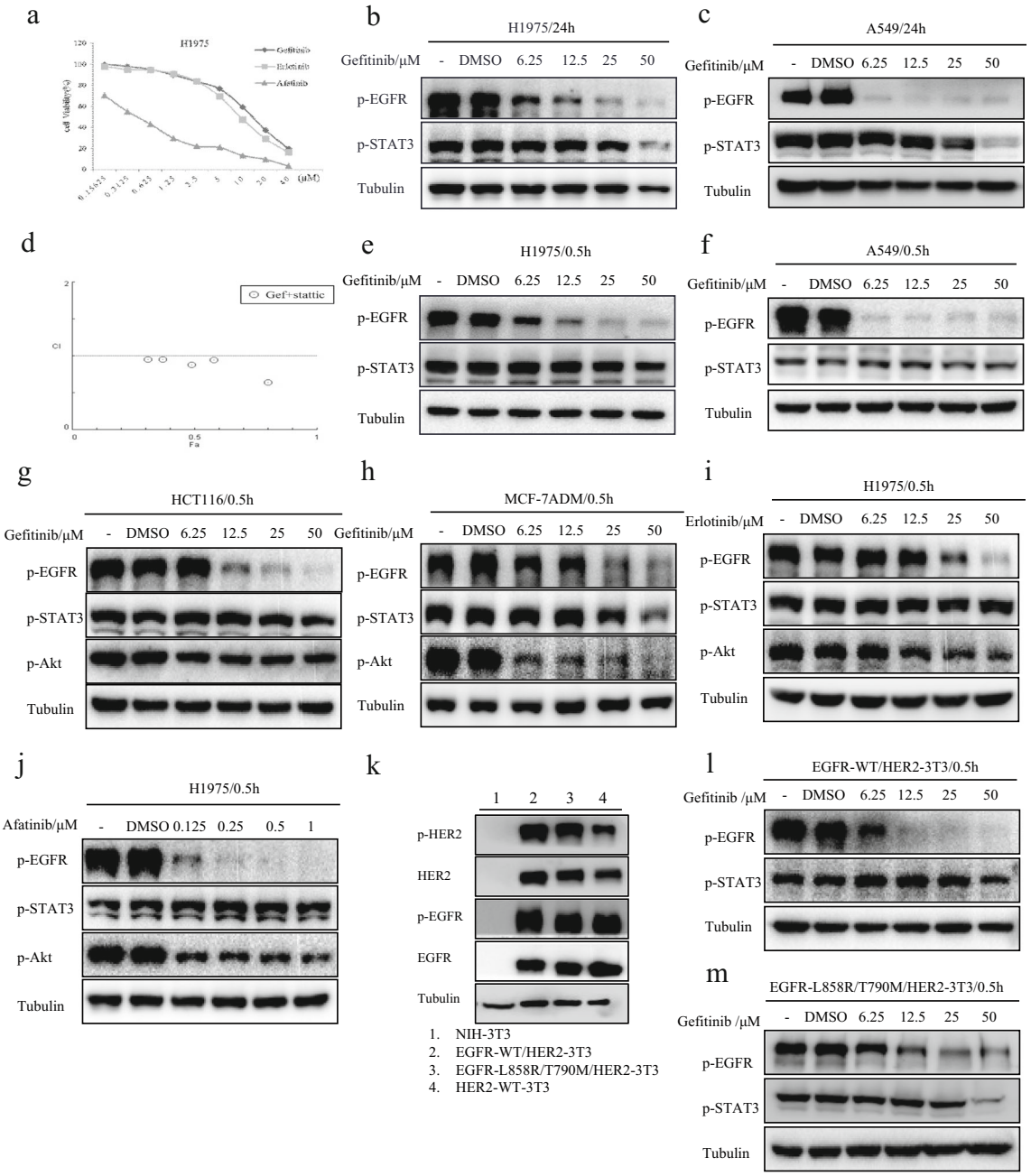

Fig. 1 Activation of STAT3 mediates resistance to gefitinib in NSCLC cells. **a** H1975 cells were treated with serial dilutions of gefitinib, erlotinib or afatinib for 72 hours. The cell viability was measured by MTT assay. **b, c** H1975 or A549 cells were treated by gefitinib (6.25 μM, 12.5 μM, 25 μM, 50 μM) for 24 hours, cells were harvested for Western blot analysis. Tubulin was used as a loading control. d Gefitinib in combination with stattic induces the proliferation inhibition H1975 cells by the MTT assay (The CI value was calculated according to the CompuSyn software, $n \geq 3$). The $CI > 1$ is antagonistic, $CI = 1$ is additive and $CI < 1$ is synergistic. **e, f** H1975 or A549 cells were treated by gefitinib (6.25 μM, 12.5 μM, 25 μM, 50 μM) for 0.5 hour, cells were harvested for Western blot analysis. **g, h** HCT116 or MCF-7ADM cells were treated by gefitinib for 0.5 hour, cells were harvested for Western blot analysis. **i, j** H1975 cells were treated by erlotinib or afatinib for 0.5 hour, cells were harvested for Western blot analysis. **k** NIH-3T3 cells stably expressing EGFR/HER2 heterodimers were examined by Western blot analysis. **l, m** EGFR-WT/HER2-3T3 or EGFR-L858R/T790M/HER2-3T3 cells were treated by gefitinib for 0.5 hour, cells were harvested for Western blot analysis

was obviously inhibited while p-STAT3 was not reduced in H1975 cells and A549 cells, suggesting that sustained activation of STAT3 occurred regardless of the factors involved in feedback pathways after long-term gefitinib exposure.

We then assessed whether the sustained activation of STAT3 was involved in the resistance of other cancer cells to gefitinib. In colon cancer HCT116 cells (Fig. 1g), treatment with gefitinib for 0.5 hour inhibited the phosphorylation of

EGFR and the downstream signalling protein Akt but did not simultaneously block the phosphorylation of STAT3. Similar results were obtained in MCF-7ADM breast cancer cells (Fig. 1h). Next, we investigated whether sustained activation of STAT3 occurred after treatment with other EGFR-TKIs. As shown in Fig. 1i and j, treatment with erlotinib and afatinib for 0.5 hour also inhibited the phosphorylation of EGFR and the downstream Akt but did not affect STAT3 phosphorylation.

To further exclude the possible effects of other oncogene-encoded proteins in wild-type cancer cells on STAT3 activation, we transfected NIH-3T3 cells with a retroviral vector stably coexpressing HER2 and EGFR, which were corresponding to EGFR mutations observed in H1975 and A549 cells; these cells lines were named EGFR-L858R/T790M/HER2-3T3 cells and EGFR-WT/HER2-3T3 cells, respectively. NIH-3T3 cells do not endogenously express EGFR family members [10]. The expression levels of HER2 and EGFR in the stably transfected cells were significantly higher than those in NIH-3T3 cells, showing that the cell lines had been successfully constructed (Fig. 1k). In these constructed NIH-3T3 cell lines, the phosphorylation of EGFR was significantly inhibited in a dose-dependent manner after treatment with gefitinib for 0.5 hour, while downstream p-STAT3 was also not altered (Fig. 1l, m); these findings were consistent with the results in H1975 cells and A549 cells.

The results described above indicated that the activated STAT3-mediated resistance to EGFR-TKIs occurred regardless of the exposure time, TKI type, and cancer cell type, suggesting that the phenomenon of sustained activation of STAT3 upon treatment with TKIs had certain universality.

Activation of STAT3-mediated gefitinib resistance does not involve JAK or gp130

Upstream receptor tyrosine kinases or cytokine receptors activate STAT3 via JAK, and thus, JAK is a key protein that directly activates STAT3 in the JAK/STAT signalling pathway. In lung cancer cells, JAK1 and JAK2 have been reported to be mainly involved in the activation of STAT3, but other members of the JAK family, namely, JAK3 and TYK2, have not been reported to be involved in STAT3 activation [11]. However, the roles of JAK1 and JAK2 in STAT3 activation in lung cancer cells is still controversial [11–13]. Therefore, we examined the effect of TKIs on the activation of STAT3 after siRNA-mediated knockdown of JAK1 or JAK2 expression in H1975 cells. As shown in Fig. 2a, b and p-STAT3 was considerably decreased after JAK1 knockdown, but p-STAT3 was not further reduced after additional treatment with gefitinib or erlotinib for 0.5 hour. Additionally, the expression of JAK2 was decreased by siRNA-induced silencing in H1975 cells, but downstream p-STAT3 was not further decreased by the inhibition of p-EGFR by gefitinib (Fig. 2c). These results suggested that the sustained activation of STAT3 in H1975 cells was not caused by JAK1 or JAK2.

Gp130, an upstream member of the JAK kinase family, can also activate STAT3; thus, we examined the effect of gefitinib on the activation of STAT3 after gp130 was knocked down with siRNA to avoid omitting the effects of JAK3 and TYK2. As shown in Fig. 2d, p-STAT3 was decreased by the depletion of gp130, while STAT3 activation was sustained after gefitinib treatment in H1975 cells. Based on all these results, we concluded that the STAT3-mediated resistance to gefitinib was not related to the gp130/JAK signalling pathway. It was reported that gp130 not only participated in the activation of STAT3 through the gp130/JAK signalling pathway but also indirectly bound to EGFR to form the gp130/HER2/EGFR complex, resulting in the rephosphorylation-mediated activation of STAT3 [14]. Grant SL et al. [15] showed that gp130 bound to HER2 and formed a complex in prostate and breast cancer cells. Therefore, we speculated that gp130 was involved in the formation of HER2/EGFR heterodimers that mediate resistance in lung cancer cells. Thus, we used the crosslinker BS^3 to detect HER2/EGFR heterodimers *in vitro*, and as shown in Fig. 2e, the formation of dimers was significantly reduced after the knockdown of gp130 compared with the formation of dimers in the NC cells. Considering the results described above, STAT3 activation-mediated gefitinib resistance may not be associated with HER2/EGFR dimers.

Activation of STAT3 after gefitinib treatment in cell lines expressing EGFR alone

Then, we determined the effect of gefitinib on the activation of STAT3 in different transfected cells expressing EGFR alone, including EGFR-WT-3T3, EGFR-L858R-3T3 and EGFR-T790M/L858R-3T3 cells. The expression levels of EGFR and p-EGFR in the stably transfected cells were significantly higher than those in the NIH-3T3 cells, showing that the cell lines were successfully constructed (Fig. 3a). In addition, the EGFR exon 20 mutation accounts for at least 9% of all EGFR mutations and is the most common mutation after the exon 19 deletion mutation and the L858R mutation [16]. Therefore, we also constructed NIH-3T3 cell lines that stably expressed the EGFR exon 20 mutant. The EGFR-insNPG-3T3 cell lines that expressed human EGFR with the D770-N771 insNPG mutation had been previously generated and preserved in our laboratory. Then, we also used the retroviral vector to stably transfect two other cell lines with the EGFR exon 20 mutation plasmids, which were named EGFR-ASVins-3T3 cells (expressing human EGFR with the A767-V769 insert duplication) and EGFR-insH-3T3 cells (expressing human EGFR with insertion at H773-V774).

As shown in Fig. 3b and c, in the EGFR-L858R/T790M-3T3 cells and EGFR-WT-3T3 cells, p-EGFR began to decrease after exposure to gefitinib at a concentration of

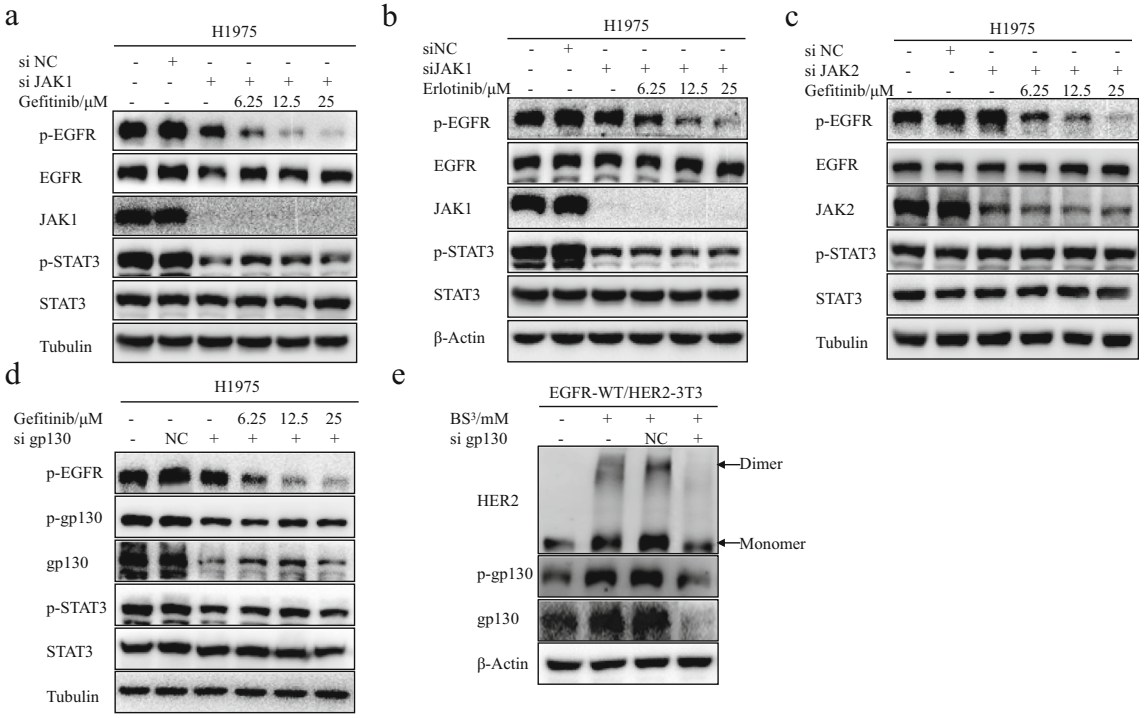

Fig. 2 Activation of STAT3-mediated gefitinib resistance does not involve JAK or gp130. **a, b** H1975 cells were transfected with 20 nmol/L of control (NC), JAK1 siRNAs for 48 hours, then cells were treated with gefitinib or erlotinib (6.25 μM, 12.5 μM, 25 μM) for 0.5 hour. **c** H1975 cells were transfected with 20 nmol/L of control (NC), JAK2 siRNAs for 48 hours, then cells were treated with gefitinib (6.25 μM, 12.5 μM, 25 μM) for 0.5 hour. Cells were harvested for Western blot analysis and proteins were examined for levels of EGFR, pY STAT3, total EGFR and STAT3, JAK1and JAK2. Tubulin was used as a loading control. Cells were harvested for Western blot analysis (top) and blots are quantified, respectively (below). **c** H1975 cells were transfected with 20 nmol/L of control, gp130 siRNAs for 48 hours, then cells were treated with gefitinib (6.25 μM, 12.5 μM, and 25 μM) for 0.5 hour. Cells were harvested for Western blot analysis. **d** EGFR-WT/HER2-3T3 cells were transfected with 20 nmol/L of control (NC), gp130 siRNAs for 48 hours, then cells were incubated with 1.0 mM BS^3 for 1 hour at room temperature. The reactions were quenched with the addition of 20 mM Tris (pH 7.4) for 15 minutes and cells were then lysed with 1 × Loading Buffer and immunoblotted to detect the indicated proteins

6.25 μM for 0.5 hour, but p-STAT3 was not inhibited. In the EGFR-L858R-3T3 cells (Fig. 3d), which were sensitive to gefitinib, p-EGFR was dramatically inhibited by gefitinib at the low concentration of 6.26 μM, but downstream p-STAT3 was not decreased. Unexpectedly, p-STAT3 was inhibited and p-EGFR was decreased in the EGFR-insNPG-3T3 cells (Fig. 4e); however, in the EGFR-insASV-3T3 cells (Fig. 3f) and EGFR-insH-3T3 cells (Fig. 3g), which also expressed EGFR exon 20 mutants, p-STAT3 did not show a sharp decline.

Comparative analysis of the three cell lines harbouring the exon 20 mutant and the EGFR-insNPG-3T3 cells that were previously constructed and preserved in our laboratory was performed. We found that NIH-3T3 cells using the same mutant EGFR construct had different TKI responses. To confirm the different findings of sustained STAT3 activation, we re-constructed the NIH-3T3 cells stably expressing EGFR-insNPG using the 3T3 cells from the same source, and the newly constructed cells were named EGFR-insNPG*-3T3 cells. As shown in Fig. 3h, p-STAT3 was not reduced, but p-EGFR was decreased, after gefitinib treatment of the newly constructed EGFR-insNPG*-3T3 cells, which was in contrast to the results in the EGFR-insNPG-3T3 cells. We speculate it may be due to the use of NIH-3T3 cells from different sources. The EGFR-insNPG-3T3 cells were constructed using 3T3 cells derived from Balb/C mice in the early years and preserved in our laboratory, while EGFR-insASV-3T3 cells and EGFR-insH-3T3 cells were recently constructed using 3T3 cells derived from Swiss mouse.

STAT3 activation-mediated gefitinib resistance is related to the expression and activation of STAT3

We then compared the differences in protein expression and activation in several cell lines using Western blotting. As shown in Fig. 4a, compared to those in the EGFR-insNPG-3T3 cells, the expression levels of STAT3 and p-STAT3 were higher in the other cell lines, prompting us to hypothesize that the gefitinib resistance mediated by STAT3 was associated with the expression and activation levels of STAT3 in the cells.

To test this hypothesis, we then depleted STAT3 by siRNA-mediated knockdown. As shown in Fig. 4b and c, after STAT3 depletion, p-STAT3 was further reduced in H1975

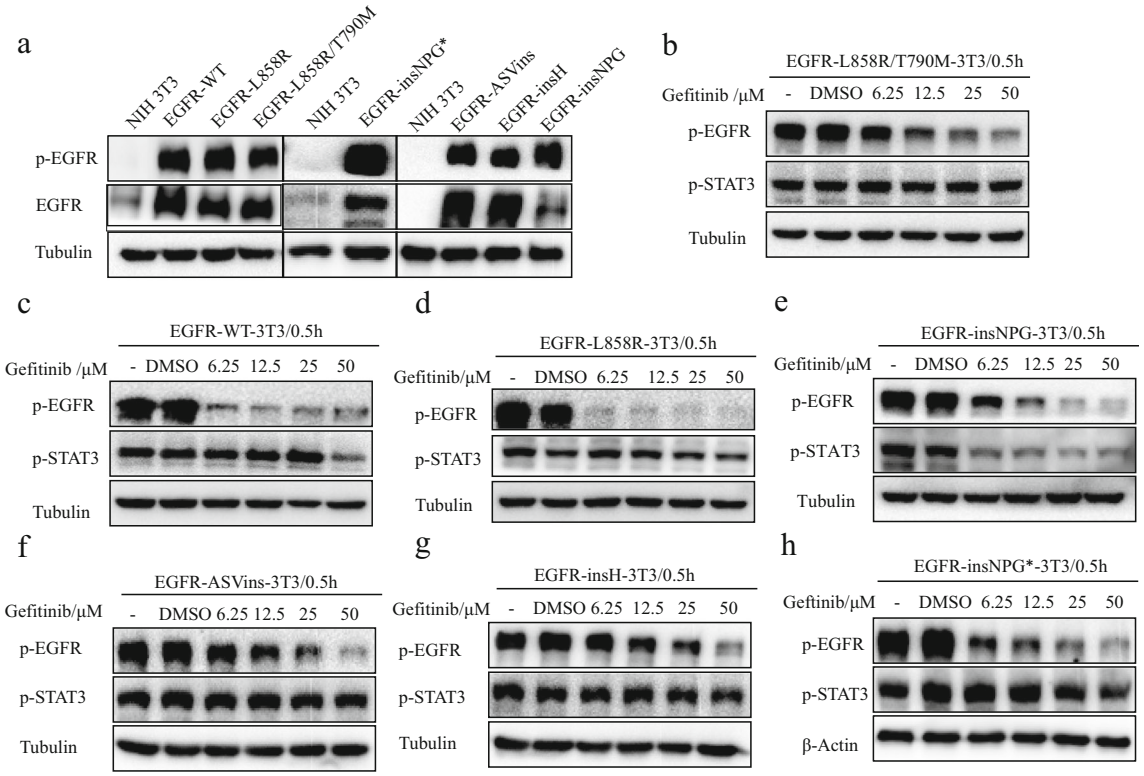

Fig. 3 Activation of STAT3 after gefitinib in cell lines expressing only EGFR. **a** Constructed NIH-3T3 cells stably expressing EGFR-WT and EGFR mutants were examined by Western blot analysis. **b-g** Different constructed cells expressing EGFR only, including EGFR-T790M/L858R-3T3, EGFR-WT-3T3, EGFR-L858R-3T3, EGFR-insNPG-3T3, EGFR-ASVins-3T3 and EGFR-insH-3T3 cells, were treated with gefitinib (6.25 μM, 12.5 μM, 25 μM and 50 μM) for 0.5 hour. Then cells were harvested for Western blot analysis and proteins were examined for levels of pY EGFR, pY STAT3. Tubulin was used as a loading control. **h** pY-STAT3 was examined after gefitinib treatment in the EGFR-insNPG*-3T3 cells which were stably transfected EGFR-insNPG plasmid in the NIH-3T3 cells

cells and EGFR-L858R/T790M-3T3 cells after gefitinib treatment for 0.5 hour. Similar results were also observed in the A549 cells, EGFR-WT-3T3 cells and EGFR-insNPG*-3T3 cells (Fig. 4d, e and f). Stattic, the first non-peptide STAT3-specific small molecule inhibitor, inhibits STAT3 dimerization and thereby inhibits STAT3 activation and nuclear translocation. As shown in Fig. 4g, p-STAT3 was significantly inhibited in H1975 cells in a dose-dependent manner after incubation with stattic and different concentrations of gefitinib for 0.5 hour. Then, we overexpressed STAT3 by transfecting a recombinant STAT3 expression plasmid into the EGFR-insNPG-3T3 cells (Fig. 4h). Results showed that the decrease in p-STAT3 after gefitinib treatment was not accompanied by an obvious decrease in p-EGFR, indicating that gefitinib resistance was associated with the expression level of STAT3.

To identify the signalling molecule that activated STAT3 after gefitinib treatment, we used the CO-IP assay with a STAT3 antibody to isolate the proteins that bound to STAT3 in the presence of gefitinib with or without stattic and then observed the changes in the proteins bound to STAT3 using Coomassie blue staining. The results showed that there was an obvious reduction in protein expression at a molecular weight of approximately 130 kD after stattic treatment (Fig. 4i). We speculated that this protein was FGFR (fibroblast growth factor receptor) because FGFR, which has a molecular weight of approximately 130 kD, has been reported to activate STAT3 [17, 18]. Furthermore, FGFR protein binding to STAT3 was decreased after stattic treatment, while FGFR protein binding to STAT3 was increased after gefitinib treatment (Fig. 4j and k), suggesting that FGFR was involved in the sustained activation of STAT3 in cells treated with gefitinib.

FGFR is associated with STAT3 activation-mediated EGFR-TKI resistance

To further verify whether FGFR was involved in the resistance to TKIs mediated by the sustained activation of STAT3, we tested and compared the expression and activation levels of the FGFR protein in p-STAT3-sensitive and non-sensitive cells after gefitinib treatment. As shown in Fig. 5a, the protein expression levels of FGFR and STAT3 in the EGFR-insNPG-3T3 cells were lower than those in the EGFR-insNPG*-3T3 cells. We then determined the combined effect of gefitinib and the FGFR inhibitor PD173074 on H1975 cell viability. As shown in Fig. 5b, the IC_{50} values of gefitinib and PD173074 were 12.12 and 10.32 μM, respectively, while co-treatment with gefitinib and PD173074

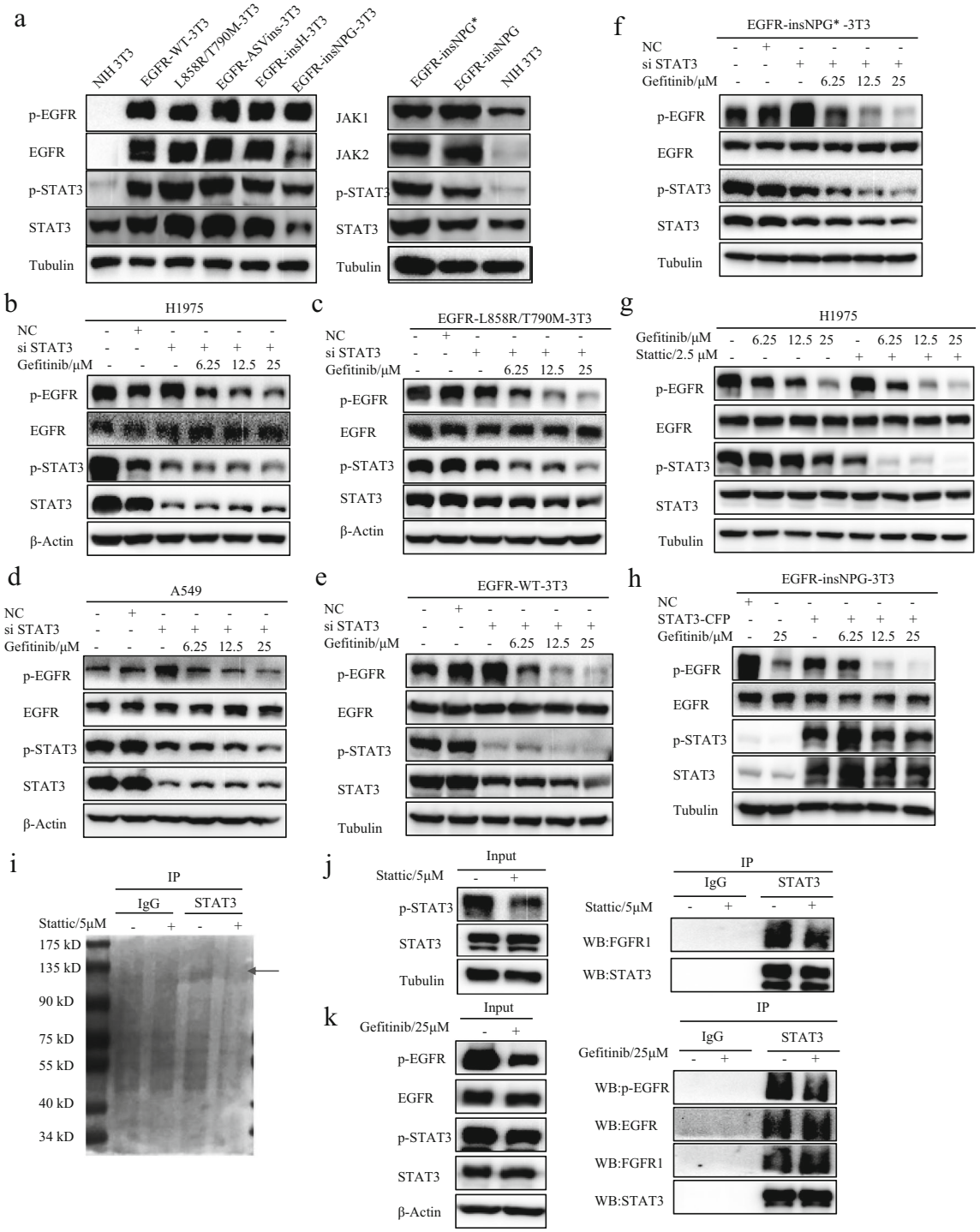

Fig. 4 STAT3 activation-mediated gefitinib resistance is related with expression and activation of STAT3. **a** Cell proteins were examined and compared for levels of pY EGFR, pY STAT3, total EGFR, STAT3, JAK1 and JAK2 in different constructed cells expressing EGFR only, including EGFR-WT-3T3, EGFR-T790M/L858R-3T3, EGFR-ASVins-3T3, EGFR-insH-3T3 cells, EGFR-insNPG-3T3 and EGFR-insNPG*-3T3 cells. **b-f** Wild NSCLC cells, including H1975(b) and A549 (d) cells, and constructed cells expressing EGFR only, including EGFR-T790M/L858R-3T3(c), EGFR-WT-3T3(e) and EGFR-insNPG*-3T3 cells(f), were transfected with 20 nmol/L of control (NC), STAT3 siRNAs for 48 hours, then cells were treated with gefitinib for 0.5 hour. Cells were harvested for Western blot analysis. **g** H1975 cells were first exposed to stattic (2.5 μM) alone for 2.5 hours, then co-incubated with gefitinib for half an hour. Cells were harvested for Western blot analysis. **h** EGFR-insNPG-3T3 cells transfected with 20 nmol/L of control (NC), STAT3-CFP plasmid for 48 hours, then cells were treated with gefitinib for 0.5 hour and harvested for Western blot analysis. **i** H1975 cells were treated by stattic (5 μM) for 3 hours, then cells were harvested for CO-IP assay and Coomassie blue staining to detect the changes of all proteins bound to STAT3. **j** H1975 cells were treated by stattic (μM) for 3 hours, then cells were harvested for CO-IP assay and Western blot to detect the changes of relevant proteins. **k** H1975 cells were treated by gefitinib (25 μM) for 0.5 hour, then cells were harvested for CO-IP assay and Western blot to detect the changes of relevant proteins

greatly enhanced the inhibition of cell proliferation (the IC_{50} value was 3.44 μM). The CI value was calculated to be 0.75 less than 1, suggesting that the combination of PD173074 and gefitinib synergistically inhibited the proliferation of H1975 cells.

Next, we examined the combined effect of gefitinib and the FGFR small molecule inhibitor PD173074 on STAT3 activation. As shown in Fig. 5c, p-FGFR was significantly inhibited, and its downstream p-STAT3 was also moderately inhibited after exposure to PD173074 alone for 8 hours. When the cells were first treated with PD173074 alone for 7.5 hours and then gefitinib was added for another half an hour, p-STAT3 was significantly inhibited in a dose-dependent manner and was almost completely suppressed at high concentrations. Similar results were observed in HCT116 colon cancer cells (Fig. 5d). Consistently, PD173074 also enhanced the potential of

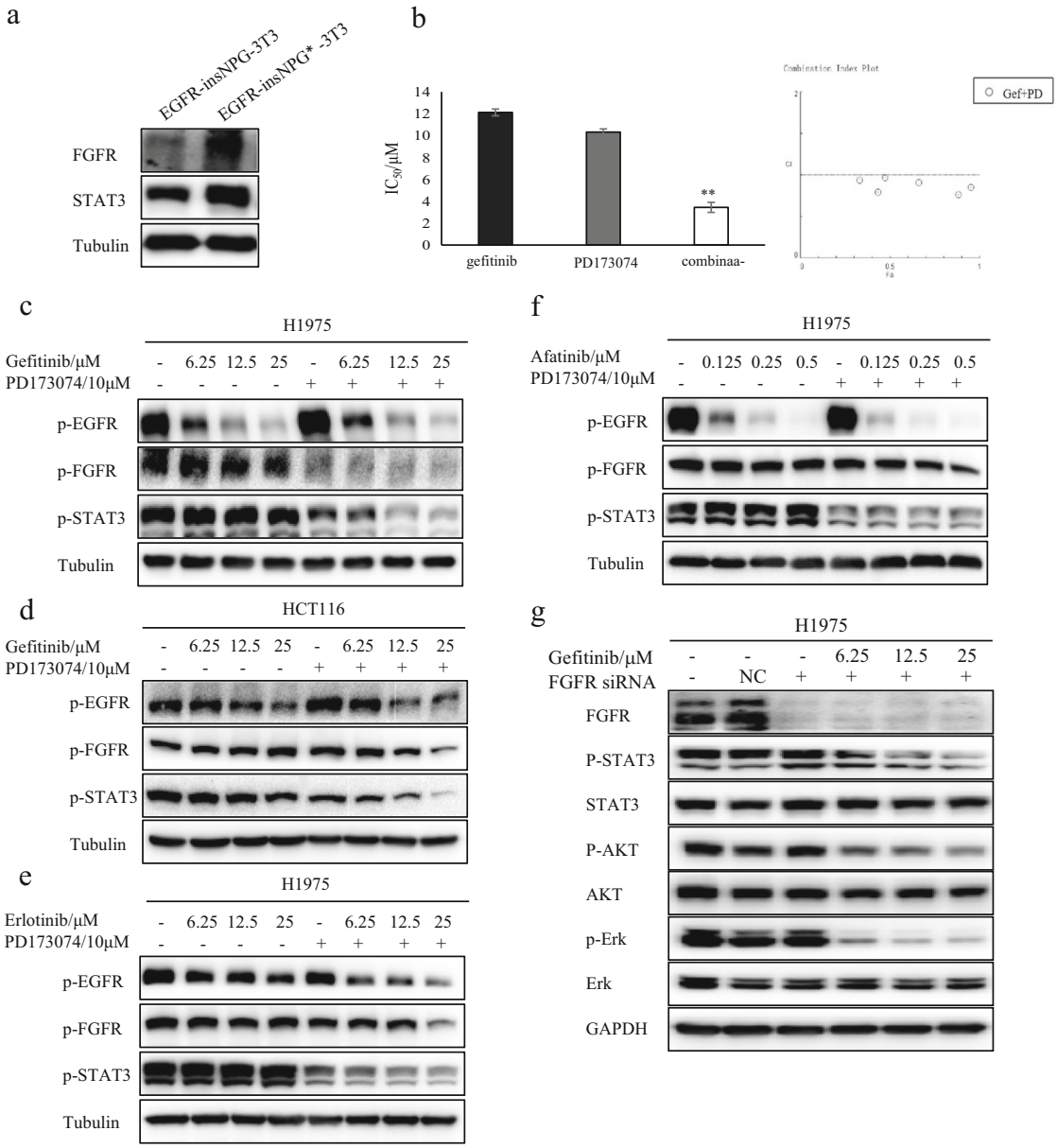

Fig. 5 FGFR is associated with activation of STAT3-mediated gefitinib resistance. **a** Cell proteins were examined and compared for levels of total FGFR and STAT3 in EGFR-insNPG-3T3 and EGFR-insNPG*-3T3 cells. **b** Gefitinib in combination with PD173074 induces the synergistic inhibition of proliferation. We examined the effects of gefitinib and PD173074, either alone or in combination, on growth inhibition of H1975 cells by the MTT assay (The CI value was calculated according to the CompuSyn software, $n \geq 3$). The $CI > 1$ is antagonistic, $CI = 1$ is additive and $CI < 1$ is synergistic. $n = 3$, $^{**}P < 0.01$, combination vs. gefitinib, $^{**}p < 0.01$, combination vs. PD173074. **c, d** H1975 or HCT116 cells were first exposed to PD173074 (10 μM) alone for 7.5 hours, then co-incubated with gefitinib for 0.5 hour. Cells were harvested for Western blot analysis. **e, f** H1975 cells were first exposed to PD173074 (10 μM) alone for 7.5 hours, then co-incubated with erlotinib (e) of afatinib (f) for 0.5 hour. Cells were harvested for Western blot analysis. **g** H1975 cells were transfected with 20 nmol/L of control (NC), FGFR siRNAs for 48 hours, then cells were treated with gefitinib for 0.5 hour. Cells were harvested for Western blot analysis

erlotinib and afatinib to inhibit the phosphorylation of STAT3 in H1975 cells (Fig. 5e and f). To exclude the possibility off-target effects of the FGFR inhibitor, we further examined the effect of gefitinib on the activation of STAT3 when FGFR was knocked down with siRNA in H1975 cells. Similar to cells treated with the FGFR inhibitor, in cells treated with FGFR-specific siRNA, p-STAT3 was decreased in a dose-dependent manner after gefitinib treatment (Fig. 5g). The above results indicate that FGFR plays an important role in the sustained activation of STAT3 after EGFR-TKI treatment.

Discussion

Oncogene addiction refers to the phenomenon of tumour cell reliance on overactive genes or signalling pathways to achieve their growth and survival, setting the stage for molecularly targeted cancer therapy [19]. Despite the remarkable efficacy of these small molecule anticancer drugs in their initial use, without exception, their use is limited by the emergence of drug resistance. Overactivation of these oncogenes not only affects the efficacy of small molecule targeted drugs but also leads to drug resistance. In addition to the secondary mutations in the targeted proteins and the emergence of alternative RTK systems, a more reasonable explanation for resistance is the notion of RTK coactivation and cross-talk network formation [18, 20].

In our paper, we found that primary resistance to EGFR-TKIs was mediated by the sustained activation of STAT3 not only in lung cancer cells but also in other tumour cells, including colon cancer cells and breast cancer cells. Moreover, continuous activation of STAT3 was also induced in EGFR mutant cells by third-generation EGFR-TKIs, such as osimertinib [7], indicating that STAT3 activation could mediate resistance to EGFR-TKIs; however, the underlying mechanism remained largely unknown. Our results showed that STAT3 continued to be activated after gefitinib treatment for only half an hour, suggesting that such primary resistance might be independent of cytokines from feedback activation pathways.

The STAT proteins can be phosphorylated and activated by many upstream kinases, including cytokine receptors and tyrosine kinases, through JAK, and the abnormal expression of the components of the JAK/STAT3 signalling pathway has been reported in gefitinib-resistant NSCLC cells [11]. The JAK family includes JAK1, JAK2, JAK3 and TYK2. It was reported that in H358 cells, inhibition of TYK2 and JAK3 with siRNA did not affect the activation of STAT3, but inhibition of JAK1 and JAK2 with siRNA resulted in the loss of STAT3 tyrosine phosphorylation and the inhibition of cell growth, indicating that JAK1 and JAK2 were mainly involved in the activation of STAT3 in lung cancer [11]. Hedavt M et al. [13] reported that inhibition of JAK2 could block the nuclear translocation of STAT3 and that the JAK2 inhibitor AZ960 resulted in the loss of STAT3 tyrosine phosphorylation in HCC827 cells, H1650 cells and other human solid tumour cells, suggesting that JAK2 mainly mediated the activation of STAT3 in these tumour cells. In addition, inhibition of JAK2 could restore erlotinib sensitivity in TKI-resistant cell lines and in transplanted tumour models, indicating that JAK2 was involved in erlotinib resistance [21]. Interestingly, Song L et al. [11] reported that inhibition of JAK2 with the specific inhibitor AG490 or RNA interference hardly inhibited the phosphorylation of STAT3 in H1975 cells and HCC827 cells and that p-STAT3 was only downregulated after knockdown of JAK1, indicating that JAK1 mediated STAT3 activation in these two tumour cells. Together, these findings suggest that the roles of JAK1 and JAK2 in the activation of STAT3 in lung cancer cells are controversial. In this study, we found that phosphorylation of STAT3 was not further inhibited after treatment with gefitinib and JAK1 or JAK2 siRNA, indicating that the STAT3-mediated gefitinib resistance in H1975 cells did not involve JAK1 and JAK2.

Gp130 is a key protein in the cytokine gp130/JAK signalling pathway and an important cytokine receptor for the activation of STAT3. Gp130 not only activates STAT3 by the phosphorylation of JAK but also induces the rephosphorylation of STAT3 by the formation of a gp130/HER2/EGFR complex [15]. We demonstrated that gp130 affected the formation of the EGFR/HER2 dimer, while STAT3 continued to be activated after gp130 knockdown. These results confirm that STAT3 activation-mediated gefitinib resistance is not associated with any member of the JAK family as molecules downstream of gp130 and suggest that EGFR/HER2 dimers may not be involved in the sustained activation of STAT3.

A previous study showed [22] that lapatinib, a reversible tyrosine kinase inhibitor of EGFR and HER2, inhibited the activation of STAT3 by blocking the heterodimerization of EGFR and HER2, thus enhancing its inhibitory effect on gefitinib-resistant cells. Moreover, the combination of lapatinib and cetuximab induced dimer dissociation and EGFR downregulation, suggesting that the formation of HER2/EGFR dimers might further activate STAT3 and participate in gefitinib resistance. However, STAT3 continued to be activated after gefitinib treatment for half an hour in transfected NIH-3T3 cells expressing wild-type or mutant EGFR alone, confirming that the sustained activation of STAT3 after treatment with gefitinib was not associated with the formation of HER2/EGFR dimers.

In our study, the inhibition or overexpression of STAT3 by genetic manipulation had the opposite effect on the sustained activation of STAT3 after gefitinib treatment. Consistent with inhibition of STAT3, the small molecule inhibitor static offset the sustained activation of STAT3 and enhanced its sensitivity in gefitinib-resistant cells, indicating that gefitinib resistance was associated with the activation levels of the STAT3 protein in cells. It was reported [23] that targeting FGFR/JAK1

inhibited the erlotinib resistance induced by STAT3 feedback activation. We also experimentally found through a CO-IP assay that the binding of FGFR to STAT3 was increased after gefitinib treatment. The combination of gefitinib and PD173074 synergistically inhibited the proliferation of H1975 cells. Moreover, FGFR inhibitors or FGFR knockdown counteracted the sustained activation of STAT3 after gefitinib treatment. In combination with PD173074, the other two EGFR TKIs, erlotinib and afatinib, also inhibited STAT3 activation, suggesting that FGFR was associated with the resistance mediated by STAT3 activation. However, our findings do not prove that any member of the JAK family is related to the STAT3-mediated resistance. The FGFR-induced phosphorylation of STAT3 may depend on other intercellular kinases, such as SRC, AKT or ERK. [24, 25], which are downstream molecules of FGFR and have been implicated in the activation of STAT3.

Conclusions

In summary, we elucidate that the FGFR/STAT3 axis, independent of JAK, participates in the sustained activation of STAT3, resulting in resistance to EGFR-TKIs during NSCLC treatment. Our study also suggests that the combination of TKIs and FGFR inhibitors is worth considering in clinical cancer treatment.

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Data availability All data generated or analysed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication The participant has consented to the submission of this report to the journal.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Code availability Not applicable.

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