



**A JAK2-related pathway induces acquired erlotinib resistance in lung cancer cells harboring an EGFR-activating mutation**

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	targeting therapy, (5-2) Signal transduction < (5) Signal transduction

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For Review

**Title****A JAK2-Related Pathway Induces Acquired Erlotinib Resistance in Lung Cancer Cells Harboring an EGFR-Activating Mutation**

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**Abstract** (245 words)

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, such as gefitinib and erlotinib, are effective for non-small cell lung cancer with activating EGFR mutations. However, even in patients with an initial dramatic response to such a drug, acquired resistance develops after 6–12 months. A secondary mutation of T790M in EGFR and amplification of the *MET* gene account for this resistance; however, the mechanism(s) of approximately 30% of acquired resistance cases remain unknown. We established an erlotinib-resistant lung cancer cell line named PC-9/ER3 that harbors an EGFR mutation after continuously exposing PC-9 cells to erlotinib. PC-9/ER3 cells were 136-fold more resistant to erlotinib than the parental cells. Although the PC-9/ER3 cells did not carry the T790M mutation or *MET* amplification and had similar levels of phosphorylated (p) STAT3, pJAK2 increased in the resistant cells. Three to 12 hours of exposure to erlotinib in both cell lines did not affect pJAK2 expression, but did result in increased pSTAT3 expression. pAkt in PC-9/ER3 cells was less suppressed than in PC-9

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6 cells, although pEGFR and pMAPK were markedly suppressed in both cell lines. The  
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10 combined treatment of erlotinib plus a JAK2 inhibitor (JSI-124) suppressed pAkt in  
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13 PC-9/ER3 cells. Similarly, the combination of erlotinib plus JSI-124 or siRNA against  
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17 JAK2 restored sensitivity to erlotinib in PC-9/ER3 cells. The combination of erlotinib  
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21 plus JSI-124 was also effective for reducing PC-9/ER3 tumors in a murine xenograft  
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25 model. Our results suggest that the activation of JAK2 partially accounts for acquired  
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29 erlotinib resistance.  
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**Introduction**

Lung cancer, the leading cause of cancer-related death in the United States, accounted for 29% of all male cancer deaths and 26% of all female cancer deaths in 2011<sup>1</sup>. The overall 5-year survival rate of patients with metastatic disease remains less than 15%<sup>2</sup>. However, somatic mutations were discovered to exist in the epidermal growth factor receptor (EGFR) tyrosine kinase in a subset of patients with non-small cell lung cancer (NSCLC)<sup>3-5</sup>. Remarkably, these mutations strongly sensitize the cancer cells to the growth suppressive effects of the EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, leading to clinical responses<sup>3, 4, 6, 7</sup>. However, the majority of NSCLCs initially sensitive to gefitinib or erlotinib become resistant to these agents within 1 year<sup>8</sup>. Once EGFR mutant lung cancer develops into progressive disease during treatment with EGFR-TKI, no optimal therapy has yet been established. Several possible mechanisms for the acquired resistance have been identified, the most common being the development of an EGFR T790M gatekeeper mutation in around 50% of cases<sup>9</sup>.

Other mechanisms of acquired resistance include bypass signaling, such as *MET* amplification<sup>10</sup>, PTEN loss<sup>11</sup>, and hepatocyte growth factor (HGF) overexpression<sup>12</sup>. Approximately 30% of cases remain for which the mechanism of acquired resistance is presently unknown<sup>9</sup>. Clinical trials testing the tolerance for changing treatment regimens to include irreversible TKI, such as BIBW2992, to prevent acquired resistance via T790M or combining EGFR-TKI with a MET inhibitor to prevent acquired resistance via amplification of *MET*, have been performed<sup>13, 14</sup>.

Phosphoinositide 3 kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription 3 (STAT3) are three major downstream pathways activated by EGFR phosphorylation<sup>15</sup>. STAT3 has been reported to be a critical mediator of the oncogenic effects of EGFR mutations<sup>16</sup>. Non-receptor tyrosine kinases, such as Src and Janus kinase 2 (JAK2), also phosphorylate STAT3<sup>17</sup>.

In this study, we isolated one cell line (PC-9/ER3) from five erlotinib-resistant clones generated *in vitro* from parental PC-9 cells that harbored the activating EGFR mutation

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by chronically exposing the cell line to erlotinib. As PC-9/ER3 cells harbored neither the T790M mutation nor *MET* gene amplification, we investigated EGFR signaling abnormalities, including those involving JAK2, STAT3, Akt, and MAPK, in these cells.

For Review



## Materials and Methods

### Establishment of an erlotinib-resistant cell line

The human NSCLC cell line PC-9 was derived from an untreated Japanese patient with pulmonary adenocarcinoma that carried an in-frame deletion in EGFR exon 19 (del E746-A750) and was highly sensitive to EGFR-TKI<sup>18</sup>. PC-9 cells were purchased from Immuno-Biological Laboratories and were cultured at 37°C with 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. To establish an erlotinib-resistant subline, the cells were treated with gradually increasing concentrations of erlotinib, starting at 0.02 µmol/L, which was near the 50% inhibitory concentration (IC<sub>50</sub>) of the drug in PC-9 cells (Fig. 1A). After 12 weeks, the cells were able to grow in 5 µmol/L erlotinib. Then, we performed single-cell cloning by limiting dilution and obtained five erlotinib-resistant cell lines.

**Sensitivity test**

Growth inhibition was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay<sup>19</sup>. Briefly, the cells were plated onto 96-well plates at a density of about  $3 \times 10^3$  cells per well and exposed to erlotinib for 96 hours. Each assay was done in quadruplicate and the mean  $\pm$  standard deviation of the IC<sub>50</sub> was calculated. Among the five erlotinib-resistant clones, the PC-9/ER3 cell line did not harbor the T790M mutation, and its resistance was stable for at least 20 weeks. Thus, we used PC-9/ER3 cells to investigate acquired resistance to erlotinib.

**Reagents and antibodies**

Erlotinib was purchased from Chemie Tek. JSI-124 (cucurbitacin I)<sup>17</sup>, a selective JAK2 inhibitor, and stattic<sup>20</sup>, an inhibitor of STAT3 activation, were acquired from Calbiochem. LY294002<sup>21</sup>, a potent inhibitor of PI3K, was obtained from Cell Signaling

Technology, Inc. Rabbit antisera against EGFR, pEGFR (pY1068), pSTAT3 (pY705), extracellular signal-regulated kinase 1/2 (ERK1/2), pERK (pT202/pY204), pAkt (pSer473), total Akt, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology. Polyclonal anti-pJAK2 (pY1007/1008) antibody was obtained from Millipore Corporation. Other polyclonal antibodies against JAK2, STAT3, survivin, and c-MYC were purchased from Santa Cruz Biotechnology.

### Sequencing of the *EGFR* gene

To determine the *EGFR* sequence, DNA was extracted from each cell line using a QIAamp DNA Mini Kit (Qiagen), and the exons encoding the intracellular domain (exons 18–22) were amplified by polymerase chain reaction (PCR). Primer sequences and amplification conditions were as described previously<sup>5</sup>. PCR products were processed with a BigDye Terminator Cycle sequencing kit (Applied Biosystems) and analyzed in both the sense and antisense directions for the presence of mutations on an

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ABI 3100 sequencer (Applied Biosystems).

**Quantitative PCR**

Quantitative PCR was performed on a GeneAmp 5700 (Applied Biosystems). The copy number ratio of *MET* to *GAPDH*, a housekeeping gene, was calculated using a genomic DNA sample. The sequences of the Taqman probe and primers for *MET* and *GAPDH* were as follows: human *MET*, 5'-FAM-TGCCTGCGAAGTGAAGGGTCTCCG-TAMRA-3' (Taqman probe), 5'-CCAATTTCTGACCGAGGGAATC-3' (forward primer), and 5'-GTCCTACCAT - ACATGAAACATGGA-3' (reverse primer); human *GAPDH*, 5'-FAM-TCAAGGTGGGGAGGGAGGTAGAGGGG-TAMRA-3' (Taqman probe), 5'-GGCTCCCACCTTTCTCATCC-3' (forward primer), and 5'-GATGTGGGGAGTACGCTGC-3' (reverse primer).

### Western blot analysis

Cells were lysed with radioimmunoprecipitation assay buffer [1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM  $\beta$ -glycerol-phosphate, 10 mM NaF, and 1 mM Na-orthovanadate] containing the respective protease inhibitor tablet (Roche). Whole cell lysates were separated with SDS–polyacrylamide gel electrophoresis (PAGE) and then transferred to a membrane and detected by antibodies using ECL Plus Western blot detection reagents (GE Healthcare Biosciences). Each protein was incubated with an appropriate primary antibody and detected by horseradish peroxidase (HRP)-mediated chemiluminescence (ECL Plus).

### Xenograft model

Female BALB/c nu/nu mice at 7 weeks of age were purchased from Japan Charles River Co. All mice were provided with sterilized food and water and housed in a barrier

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facility under a 12-h light/dark cycle. Cells ( $2 \times 10^6$ ) were injected subcutaneously (s.c.) into the backs of the mice. At 1 week after injection, the mice were randomly assigned to one of four groups (five to six mice per group) that received vehicle, 25 mg/kg/day erlotinib, 1 mg/kg/day JSI-124, or 25 mg/kg/day erlotinib plus 1 mg/kg/day JSI-124. Vehicle and erlotinib were administered once a day, five times a week by gavage. JSI-124 (1 mg/kg) was administered once a day, five times a week intraperitoneally (i.p.). Tumor volume ( $\text{width}^2 \times \text{length}/2$ ) was determined periodically.

All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee of the Department of Animal Resources, Okayama University Advanced Science Research.

**siRNA gene knockdown**

MISSION predesigned small interfering (si)RNAs (Sigma) targeting STAT3 and JAK2 sequences were 5'-GGAUAACGUCAUUAGCAGA[dT][dT]-3' and 5'-

UCUGCUAAUGACGUUAUCC[dT][dT]-3' and

5'-GAUAGGUGCCCUAGGGUUU[dT][dT]-3' and

5'-AAACCCUAGGGCACCUAUC[dT][dT]-3', respectively. MISSION siRNA

Universal Negative Control (Sigma) was used as a non-targeting control for siRNA

experiments. PC-9 and PC-9/ER3 cells were transiently transfected with the

combination of two siRNA duplexes (5 nM STAT3-specific siRNA and 30 nM

JAK2-specific siRNA) using Lipofectamine 2000 (Invitrogen) according to the

manufacturer's protocol. Assays for silencing were performed on confluent monolayers

24 or 36 hours after transfection. Protein expression after siRNA knockdown was

evaluated by Western blot analysis. Proliferation of PC-9/ER3 and PC-9 cells, in which

STAT3 or JAK2 was knocked down by siRNA, was measured using the MTT assay as

described above.

## Statistical analysis

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The differences between the groups were compared using Student's t-test.  $P < 0.05$  was considered statistically significant. All data were analyzed using Microsoft Office Excel 2007 (Microsoft Japan Corporation, Tokyo).

For Review



## Results

### PC-9/ER3 cells were established after continuous exposure of PC-9 cells to erlotinib

By microscopic observation, PC-9/ER3 cells showed the same morphology as the parental PC-9 cells (Fig. 1B). The rate of proliferation of PC-9/ER3 cells was similar to that of PC-9 cells, with doubling times of 13.3 and 17.5 hours ( $P = 0.43$ ), respectively (Fig. 1C). The parental PC-9 cells could not proliferate in the presence of 1  $\mu\text{mol/L}$  erlotinib, whereas PC-9/ER3 cells continued to grow under the same condition (Fig. 1B). The  $\text{IC}_{50}$  values of erlotinib in the PC-9 and the PC-9/ER3 cells were  $0.0089 \pm 0.0001$  and  $1.21 \pm 0.11 \mu\text{mol/L}$ , respectively. PC-9/ER3 cells were 136-fold more resistant to erlotinib than the parental PC-9 cells. The resistance was stable for at least 20 weeks without exposure to erlotinib (Fig. 1D). The  $\text{IC}_{50}$  values for gefitinib were  $0.011 \pm 0.001 \mu\text{mol/L}$  in PC-9 cells and  $2.78 \pm 0.42 \mu\text{mol/L}$  in PC-9/ER3 cells, indicating that

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PC-9/ER3 cells showed cross-resistance to gefitinib that was 252-fold higher than in PC-9 cells. Both PC-9/ER4 and PC-9/ER5 cells were also resistant to erlotinib and gefitinib (Supplemental Fig. 1).

**Mechanism of erlotinib resistance is not due to the T790M point mutation or *MET* gene amplification**

To examine genetic alterations, including the well-known T790M mutation, we conducted direct sequencing of *EGFR* at exons 18–22. The T790M mutation at exon 20 was not observed in the PC-9/ER3 cells (Fig. 2A). No other genetic differences in *EGFR* DNA sequences<sup>22</sup> between PC-9 and PC-9/ER3 cells were detected. Next, we used a more sensitive assay for the *EGFR* T790M mutation: the peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp-based detection test (Mitsubishi Chemical Medience Corp.), which can detect mutations present in 0.1% to 1% of samples<sup>23, 24</sup>. This method also failed to detect the T790M mutation in PC-9/ER3 cells,

confirming the results of direct sequencing. In addition, the PCR-Invader method (Bio Medical Laboratories), which was more sensitive than the direct sequencing and was as sensitive as PNA-LNA PCR clamp-based detection test<sup>25</sup>, also failed to detect T790M in the other resistant cell lines (PC-9/ER1, 2, 3, 4 and 5).

The second most common cause of acquired resistance of NSCLC to EGFR-TKIs *in vitro* and *in vivo* involves the amplification of *MET*<sup>10, 26, 27</sup>. We examined differences in *MET* amplification between PC-9 and PC-9/ER1, 2, 3, 4 and 5 cells using a quantitative PCR method. No *MET* gene amplification was detected in PC-9/ER3 cells (Fig. 2B) or the other resistant cells (PC-9/ER1, 2, 4 and 5) (Supplemental Fig. 2). In addition, PTEN expression was similar in both PC-9 and PC-9/ER3 cell lines (Fig. 2C).

### **pJAK2 increased in PC-9/ER3 cells**

PC-9 and PC-9/ER3 cells were treated with erlotinib (0.05  $\mu$ M) for various lengths of time (0–12 hours). pEGFR and pMAPK in both cell lines were markedly suppressed by erlotinib. However, pAkt, which is an effector molecule downstream of EGFR, was not

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inhibited by erlotinib, and pJAK2 increased in PC-9/ER3 more than in the parental PC-9 cells (Fig. 3). JAK2 in both PC-9/ER4 and PC-9/ER5 cells was also activated (Supplemental Fig. 3). Phosphorylation of STAT3, which is a downstream effector of JAK2, increased time-dependently in both PC-9 and PC-9/ER3 cells after treatment with erlotinib. Although expression of survivin, the anti-apoptotic gene that is downstream of STAT3, did not change during erlotinib treatment, PC-9/ER3 cells had more expressions than PC-9 cells as a base line (Fig. 3).

**Inhibition of JAK2 rather than STAT3 restores the sensitivity of PC-9/ER3 cells to erlotinib**

We hypothesized that the inhibition of JAK2/STAT3 or PI3K/Akt might restore sensitivity to erlotinib in PC-9/ER3 cells. The differences in sensitivity to treatment with erlotinib plus or minus JSI-124, LY294002, or stattic were evaluated using the MTT assay (Fig. 4A–F). JSI-124, LY294002 and stattic suppressed pJAK2, pAkt and pSTAT3, respectively, in both PC-9 and PC-9/ER3 cells (Fig. 5 and Supplemental Fig. 4). Cells were treated with the indicated concentration of erlotinib in combination with the

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6 approximate IC<sub>50</sub> concentration of each drug (0.05  $\mu$ M JSI-124, 15  $\mu$ M LY294002, or 5  
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10  $\mu$ M static) for 96 hours. The respective sensitivities of PC-9 and PC-9/ER3 cells to  
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13 JSI-124 alone were quite similar (Fig. 4A). The sensitivity of the PC-9/ER3 cells to  
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17 erlotinib was restored by the combined treatment of erlotinib plus JSI-124 to a level  
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21 comparable to that of PC-9, while the sensitivity of PC-9 cells to erlotinib did not  
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25 increase with the same treatment (Fig. 4B). The sensitivity of PC-9/ER3 cells to  
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29 LY294002 was slightly higher than that of PC-9 cells (Fig. 4C). The erlotinib sensitivity  
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33 of PC-9/ER3 cells was moderately restored upon treatment with erlotinib plus  
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37 LY294002, but that of PC-9 cells was not affected (Fig. 4D). The sensitivity of both cell  
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41 lines to static was similar (Fig. 4E). However, the sensitivity of PC-9/ER3 cells to  
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45 erlotinib was nearly unaffected by the presence of static, while the sensitivity of the  
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49 PC-9 cells to erlotinib in the presence of static decreased (Fig. 4F).

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51 To confirm the interaction of erlotinib (0.01  $\mu$ M) with JSI-124 (0.03  $\mu$ M), LY294002  
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55 (5  $\mu$ M), or static (2  $\mu$ M), the number of viable cells after drug exposure for 96 hours  
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was counted. As shown in Figure 4G, the growth inhibition ratio after treatment with erlotinib plus JSI-124 of PC-9/ER3 cells increased significantly ( $P < 0.05$ ). That was also proved in PC-9/ER5 cells (Supplemental Fig. 5). However, the combination of erlotinib with LY294002 or stattic was not effective in the resistant cells.

These results suggest that the combination of erlotinib with a JAK2 inhibitor was more effective than the combination of erlotinib with a STAT3 inhibitor in PC-9/ER3 cells.

**The combination of erlotinib with JSI-124 suppresses pAkt in PC-9/ER3 cells**

To determine whether the combined effect of erlotinib with JSI-124 correlated with changes in MAPK or Akt signaling pathways, cells were treated with erlotinib alone, JSI-124 alone, or the combination of both drugs, and the lysates were processed for Western blotting with antibodies specific for pJAK2, pSTAT3, pAkt, and pMAPK.

Figure 5 shows that treatment with erlotinib alone inhibited pMAPK in both cell lines as

well as pAkt in PC-9, but not in PC-9/ER3 cells. We used 10  $\mu$ M of JSI-124, which seemed high compared to the results from MTT assays. However, treatment duration of the drug was 6 hours in Western blot analysis and 96 hours in MTT assays. The concentration of JSI-124 was referred from the report of Blaskovich et al <sup>17</sup>. Treatment with JSI-124 alone inhibited pJAK2, pSTAT3, and pMAPK in both cell lines as well as pAkt in PC-9/ER3 cells, but not in PC-9 cells. As expected, the phosphorylation of Akt in PC-9 cells treated with both erlotinib and JSI-124 was similar to that with erlotinib alone. However, in PC-9/ER3 cells, JSI-124 moderately suppressed pAkt. Additionally, the combination of both drugs suppressed pAkt as much as that detected in PC-9 cells treated with erlotinib alone. The combination of si-JAK2 with erlotinib suppressed pAkt in PC-9/ER3 cells (Supplemental Fig. 6).

#### **siRNA knockdown of JAK2 also recovers sensitivity to erlotinib in PC-9/ER3 cells**

We examined whether siRNA knockdown of *STAT3* or *JAK2* would restore the

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sensitivity to erlotinib in PC-9/ER3 cells (Fig. 6). Accordingly, gene-specific siRNAs were transfected into PC-9 and PC-9/ER3 cells. The mock (control) transfected cells received transfection reagents without siRNA. siRNA specific for *STAT3* and *JAK2* lowered *STAT3* and *JAK2* expression, respectively (Fig. 6A and C). Subsequently, we evaluated the growth inhibition of each cell line by erlotinib using MTT assays. The knockdown of *STAT3* did not increase the sensitivity to erlotinib of either cell line (Fig. 6B). The knockdown of *JAK2* did not influence the sensitivity of PC-9 cells to erlotinib, but it did restore the sensitivity of PC-9/ER3 cells to erlotinib (Fig. 6D). These results suggest that the resistance of PC-9/ER3 cells to erlotinib was not induced by the activation of the JAK2/STAT3 pathway, but rather a JAK2-related pathway, such as the PI3K/Akt pathway.

**The combination of erlotinib with JSI-124 to treat PC-9/ER3 tumors in a xenograft model is effective**



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6 The antitumor effect of the combination of erlotinib with JSI-124 was examined *in*  
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10 *vivo*. PC-9/ER3 xenograft model mice were treated with JSI-124 alone, erlotinib alone,  
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13 erlotinib in combination with JSI-124, or vehicle alone (Fig. 7). JSI-124 alone did not  
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17 inhibit tumor growth. Erlotinib alone did not significantly affect tumor volume. The  
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25 alone ( $P < 0.05$ ). These results showed that the combination of erlotinib and JSI-124  
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29 restored the sensitivity of PC-9/ER3 cells to erlotinib *in vivo*.  
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**Discussion**

Several EGFR-related proteins were evaluated to identify the signal abnormalities of erlotinib-resistant PC-9/ER3 cells, which had JAK2 activation without STAT3 activation. We found that JAK2 phosphorylation followed by the activation of Akt partially accounted for acquired erlotinib resistance and that resistance could be overcome by treatment with a combination of erlotinib and a JAK2 inhibitor.

The STAT family of transcription factors consists of seven proteins in humans (STAT1–STAT4, STAT5A, STAT5B, and STAT6) that are encoded by separate genes. STAT3 and STAT5 are the STATs most often implicated in human cancer progression<sup>28</sup>. Activated STAT3 and STAT5 were expressed in about 55% and 33% of NSCLC tumors, respectively<sup>29, 30</sup>. STAT3 has been the subject of more investigations than STAT5<sup>28</sup>.

Our previous study showed that pSTAT3 was less suppressed compared to EGFR despite the administration of gefitinib in our mutant EGFR-transgenic mice. It suggests

that signals from other upstream might activate STAT3 even in EGFR-driven lung cancer<sup>31</sup>. Although we focused on STAT3 in this study, STAT5 should be further examined in our future experiment. Aberrant STAT3 activation was shown to be required for the survival of human cancer cells by promoting the overexpression of genes that encode anti-apoptotic proteins, cell-cycle regulators, and angiogenic factors<sup>32-34</sup>. STAT activation by cytokines is mediated through JAKs, which include four family members, JAK1, JAK2, JAK3, and Tyk2<sup>35</sup>. In PC-9/ER3 cells, STAT3 did not seem to play a critical role in erlotinib resistance. Activation of Akt was inhibited by blocking activation of JAK2 in PC-9/ER3 cells (Fig. 5). Although a direct relationship between the JAK2 and Akt pathways remains unclear, our data indicated a connection. Vogt and Hart supposed that two branches of oncogenic signal initiated by PI3K (Akt-mTOR and BMX-STAT3 pathways) were networked<sup>36</sup>. Although it has never been proved, Akt from JAK2 axis in EGFR resistance might emerge in the network.

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Recent studies in breast cancer, lung cancer, and diffuse large B cell lymphoma cell lines have demonstrated a central role for JAK family kinases in mediating IL-6 signaling in these cells<sup>29, 37, 38</sup>. Our study provides a molecular reason for JAK2 activation and highlights JAK2 as one of several mechanisms of acquired drug resistance and a potential target for recovery from resistance. No difference was observed in IL-6 protein levels evaluated by enzyme-linked immunosorbent assay between PC-9 and PC-9/ER3 cells (data not shown). Lee et al. reported that JAK1 and JAK2 activation participates in IL-5-induced upregulation of *c-MYC* in a human hematopoietic progenitor cell line<sup>39</sup>. In Bcr-Abl<sup>+</sup> chronic myelogenous leukemia cells, the activation of JAK2 did not lead to STAT5 activation, which was activated by Bcr-Abl<sup>40, 41</sup>. One major effect of the activation of JAK2 by the Bcr-Abl oncoprotein is increased *c-MYC* expression, which is required for leukemia induction<sup>40-43</sup>. Inhibition of JAK2 resulted in decreased pAkt and c-MYC in imatinib-resistant chronic myelogenous leukemia cells, and JAK2 was identified as a potentially important

therapeutic target for imatinib-resistant chronic myelogenous leukemia<sup>43</sup>. *MYC* is a classical oncogene in lung cancer, and its amplification in adenocarcinoma of the lung occurs in both late and early stages of lung cancer progression and serves as a prognostic molecular marker<sup>44</sup>. Thus, we expected that inhibition of JAK2 would result in decreased pAkt and c-MYC, which would lead to PC-9/ER3 cell death independently of STAT3 suppression. In reality, *JAK2*-specific siRNA inhibited c-MYC expression, and the combination of erlotinib and JSI-124 efficiently suppressed c-MYC expression in PC-9/ER3 cells (Supplemental Fig. 7). The relationships among JAK2, Akt, and MYC as downstream signals of EGFR in erlotinib-resistant lung cancer cells should be pursued further.

Our study had some limitations. Only one cell line (PC-9/ER3) was extensively investigated. The activation of JAK2 was also considered to be one of the various EGFR-TKI-resistant mechanisms in the other resistant line (PC-9/ER5) derived from PC-9. However, this mechanism may be applied only to PC-9. We should establish

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further EGFR-TKI-resistant cell lines derived from other sensitive cell lines such as HCC827 and H3255, and need to verify whether activated JAK2 was really related with the resistant mechanism. Additionally, the resistant cells were 136-fold more resistant to erlotinib than the parental cells, which is not easily translatable to a clinical situation. However, such a big difference in sensitivity to erlotinib between the cells may have helped us identify a new mechanism of resistance. Patients with NSCLC who underwent surgery and had high pJAK2 expression had a significantly worse overall survival rate compared with those with low pJAK2 expression <sup>45</sup>. Future studies should examine pJAK2 expression in EGFR-TKI-resistant clinical samples.

Unexpectedly, PC-9/ER3 cells did not carry the T790M mutation, although PC-9 cells easily developed the T790M mutation after continuous exposure to gefitinib <sup>46</sup> or vandetanib <sup>47</sup>. Five (12.8%) of 39 erlotinib-resistant tumors in mutated EGFR-driven transgenic mice expressed the T790M mutation <sup>48</sup>. The reason why PC-9/ER3 cells

selectively bypassed JAK2 signaling but did not develop the T790M mutation or *MET* overexpression remains unknown.

In conclusion, we were able to show participation of the JAK2 pathway as one of the mechanisms of acquired erlotinib resistance and that acquired erlotinib resistance could be overcome by suppression of the JAK2 pathway both *in vitro* and *in vivo*.

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### Disclosure Statement

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## Figure Legends

**Figure 1.** Establishment of an erlotinib-resistant lung cancer cell line.

A, Overview of the strategy to establish erlotinib-resistant cells from PC-9 cells. B, A

light microscopic ( $\times 100$ ) image of PC-9 and PC-9/ER3 cells. Cells were exposed to

erlotinib ( $1\text{ }\mu\text{M}$ ) for 72 hours. C, Relative cell numbers of PC-9 and PC-9/ER3 are

shown in the culture medium for 72 hours. PC-9 and PC-9/ER3 cells were seeded on

6-cm dishes ( $4 \times 10^5$  per dish) in the absence of erlotinib. Cells were trypsinized and

counted in triplicate. DT, doubling time. Data are presented as the mean  $\pm$  standard

error. D, Cells ( $3 \times 10^3$  per well) were seeded onto 96-well plates in quadruplicate and

grown in the absence or presence of the indicated concentration of erlotinib. After 96

hours, the cells were subjected to MTT assays. PC-9/ER3 cells were maintained in

culture medium without erlotinib for 5 (5W), 10 (10W), or 20 (20W) weeks.

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**Figure 2.** Characterization of PC-9/ER3 cells.

A, The T790M mutation was not found in PC-9/ER3 cells by direct sequencing. B, *MET* gene copy number was examined by quantitative PCR using genomic DNA extracted from PC-9 and PC-9/ER3 cells. *MET* gene copy number relative to *GAPDH* was measured in three independent experiments. Bars, standard deviation. C, Cells were incubated with or without 2  $\mu$ M erlotinib for 6 hours and subjected to Western blotting. pEGFR was suppressed, and PTEN expression was similar in both cell lines.

**Figure 3.** Protein expression in PC-9 and PC-9/ER3 cells treated with erlotinib for various lengths of time. pEGFR and pMAPK in both cell lines were markedly suppressed. pAkt was not inhibited by erlotinib, and pJAK2 increased in PC-9/ER3 cells more than in the parental PC-9 cells. pSTAT3 increased in both PC-9 and PC-9/ER3 cells in a time-dependent manner. Although expression of survivin did not change during erlotinib treatment,

PC-9/ER3 cells had more expressions than PC-9 cells as a base line.

**Figure 4.** Comparison of cell growth after treatment with drugs alone or in combination.

A, Cells ( $3 \times 10^3$  per well) were seeded in 96-well plates in quadruplicate and grown in the absence or presence of the indicated concentration of JSI-124. After 96 hours, the cells were subjected to MTT assays. B, Cells ( $3 \times 10^3$  per well) were seeded in 96-well plates in quadruplicate and grown in the absence or presence of the indicated concentration of erlotinib with or without 0.05  $\mu$ M JSI-124. After 96 hours, the cells were subjected to MTT assays. C and D, Same as in (A) and (B), respectively, but with LY294002. The concentration of LY290442 in (D) was 15  $\mu$ M. E and F, Same as in (A) and (B), respectively, but with stattic. The concentration of stattic in (F) was 5  $\mu$ M. G, Cells were treated with 10 nM erlotinib plus or minus 30 nM JSI-124 (left panel), 5  $\mu$ M LY294002 (center panel), or 2  $\mu$ M stattic (right panel) for 96 hours. Growth inhibition

relative to erlotinib-untreated cells with or without JSI-124, LY294002, or stattic are shown. Data are representative of two independent experiments. Bars, standard error.

**Figure 5.** Protein expression in PC-9 and PC-9/ER3 cells upon treatment with erlotinib plus or minus JSI-124.

Extracts from PC-9 and PC-9/ER3 cell lines, treated with erlotinib (0.01  $\mu$ M) or JSI-124 (10  $\mu$ M) or both for 6 hours, were subjected to Western blotting. Treatment with erlotinib alone inhibited pMAPK in both cell lines and pAkt in PC-9, but not PC-9/ER3, cells. Treatment with JSI-124 alone inhibited pJAK2, pSTAT3, and pMAPK in both cell lines and pAkt in PC-9/ER3, but not in PC-9, cells. pAkt in PC-9 cells treated with both erlotinib and JSI-124 was similar to that with erlotinib alone. However, in PC-9/ER3 cells, JSI-124 moderately suppressed pAkt. The combination of both drugs suppressed pAkt as much as that in PC-9 cells treated with erlotinib alone.



**Figure 6.** siRNA knockdown of *STAT3* and *JAK2*.

A, *STAT3* was knocked down in PC-9 and PC-9/ER3 cells by *STAT3*-specific siRNA. B, Cells ( $3 \times 10^3$  per well) were seeded in 96-well plates in quadruplicate and grown with the indicated concentration of erlotinib. After 96 hours, the cells were subjected to MTT assays. The knockdown of *STAT3* did not affect the sensitivity to erlotinib of either cell line. C, *JAK2*-specific siRNA lowered *JAK2* expression. D, The knockdown of *JAK2* did not influence the sensitivity of PC-9 cells to erlotinib, but restored the sensitivity of PC-9/ER3 cells to erlotinib.

**Figure 7.** Growth curves of PC-9/ER3 xenograft tumors.

PC-9/ER3 xenograft model mice were treated with JSI-124 (1 mg/kg/day) alone, erlotinib (25 mg/kg/day) alone, erlotinib in combination with JSI-124, or vehicle alone. JSI-124 alone did not inhibit tumor growth. Erlotinib alone did not significantly affect the tumor volume. The two-drug combination resulted in significant tumor shrinkage

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compared to either drug alone ( $P < 0.05$ ). Differences in tumor volume were compared using Student's  $t$ -test. Bars, standard error.

For Review

## Supporting information

**Supplemental Figure 1.** IC<sub>50</sub> values of erlotinib and gefitinib in PC-9, PC-9/ER3, 4 and 5 cells determined by MTT assay.

**Supplemental Figure 2.** *MET* gene copy number relative to *GAPDH* in PC-9 and PC-9/ER1, 2, 3, 4 and 5 cells.

**Supplemental Figure 3.** Protein expression in PC-9 and PC-9/ER3, 4 and 5 cells treated with or without erlotinib for 6 hours.

**Supplemental Figure 4.** Target protein expression in PC-9 and PC-9/ER3 cells treated with each indicated concentrations of LY294002 or static.

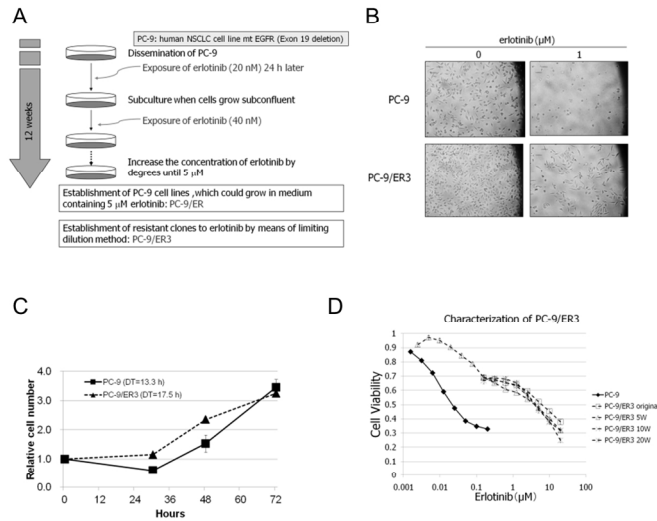
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**Supplemental Figure 5.** Growth inhibition ratio after treatment with erlotinib plus  
JSI-124 of PC-9/ER5 cells.

**Supplemental Figure 6.** The combination of erlotinib with inhibition of JAK2 using  
siRNA in PC-9/ER3 cells suppressed pAkt in PC-9/ER3 cells.

**Supplemental Figure 7.**  
Inhibition of JAK2 suppresses c-MYC expression.

Figure 1

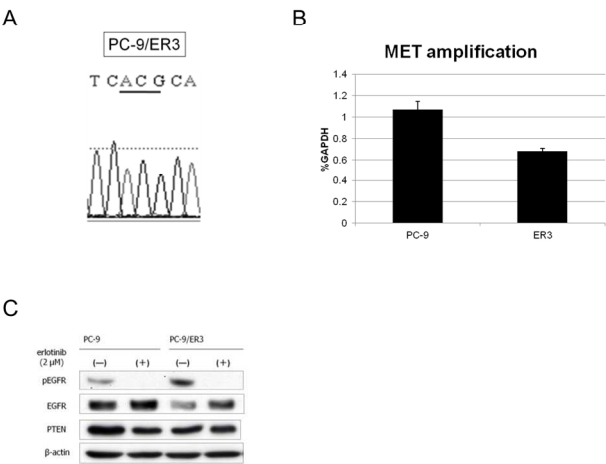


Establishment of an erlotinib-resistant lung cancer cell line.

A, Overview of the strategy to establish erlotinib-resistant cells from PC-9 cells. B, A light microscopic ( $\times 100$ ) image of PC-9 and PC-9/ER3 cells. Cells were exposed to erlotinib (1  $\mu$ M) for 72 hours. C, Relative cell numbers of PC-9 and PC-9/ER3 are shown in the culture medium for 72 hours. PC-9 and PC-9/ER3 cells were seeded on 6-cm dishes ( $4 \times 10^5$  per dish) in the absence of erlotinib. Cells were trypsinized and counted in triplicate. DT, doubling time. Data are presented as the mean  $\pm$  standard error. D, Cells ( $3 \times 10^3$  per well) were seeded onto 96-well plates in quadruplicate and grown in the absence or presence of the indicated concentration of erlotinib. After 96 hours, the cells were subjected to MTT assays. PC-9/ER3 cells were maintained in culture medium without erlotinib for 5 (5W), 10 (10W), or 20 (20W) weeks.

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Figure 2

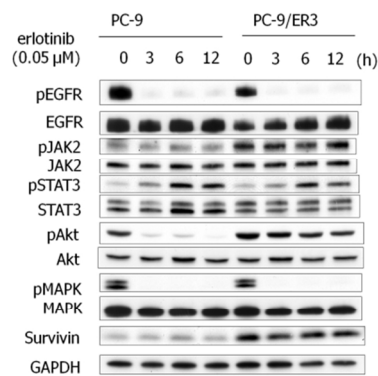


Characterization of PC-9/ER3 cells.

A, The T790M mutation was not found in PC-9/ER3 cells by direct sequencing. B, MET gene copy number was examined by quantitative PCR using genomic DNA extracted from PC-9 and PC-9/ER3 cells. MET gene copy number relative to GAPDH was measured in three independent experiments. Bars, standard deviation. C, Cells were incubated with or without 2 μM erlotinib for 6 hours and subjected to Western blotting. pEGFR was suppressed, and PTEN expression was similar in both cell lines.

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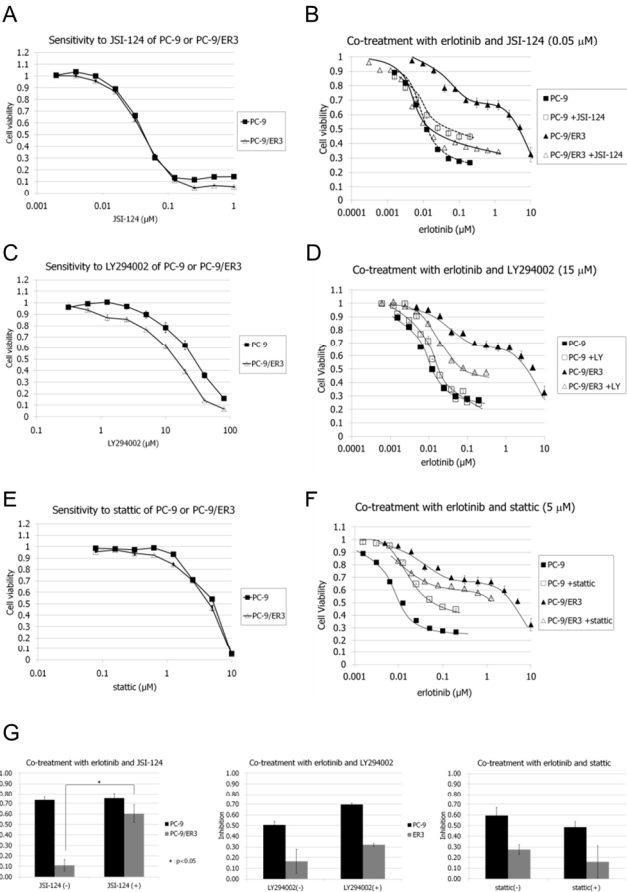
Figure 3



Protein expression in PC-9 and PC-9/ER3 cells treated with erlotinib for various lengths of time. pEGFR and pMAPK in both cell lines were markedly suppressed. pAkt was not inhibited by erlotinib, and pJAK2 increased in PC-9/ER3 cells more than in the parental PC-9 cells. pSTAT3 increased in both PC-9 and PC-9/ER3 cells in a time-dependent manner. Although expression of survivin did not change during erlotinib treatment, PC-9/ER3 cells had more expressions than PC-9 cells as a base line.

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Figure 4



Comparison of cell growth after treatment with drugs alone or in combination.

A, Cells ( $3 \times 10^3$  per well) were seeded in 96-well plates in quadruplicate and grown in the absence or presence of the indicated concentration of JSI-124. After 96 hours, the cells were subjected to MTT assays. B, Cells ( $3 \times 10^3$  per well) were seeded in 96-well plates in quadruplicate and grown in the absence or presence of the indicated concentration of erlotinib with or without 0.05  $\mu$ M JSI-124. After 96 hours, the cells were subjected to MTT assays. C and D, Same as in (A) and (B), respectively, but with LY294002. The concentration of LY294042 in (D) was 15  $\mu$ M. E and F, Same as in (A) and (B), respectively, but with stattic. The concentration of stattic in (F) was 5  $\mu$ M. G, Cells were treated with 10 nM erlotinib plus or minus 30 nM JSI-124 (left panel), 5  $\mu$ M LY294002 (center panel), or 2  $\mu$ M stattic (right panel) for 96 hours. Growth inhibition relative to erlotinib-untreated cells with or without JSI-124, LY294002, or stattic are shown. Data are representative of two independent experiments. Bars, standard error.

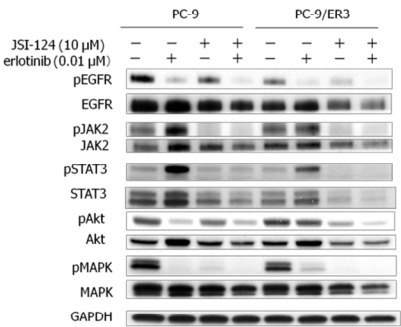
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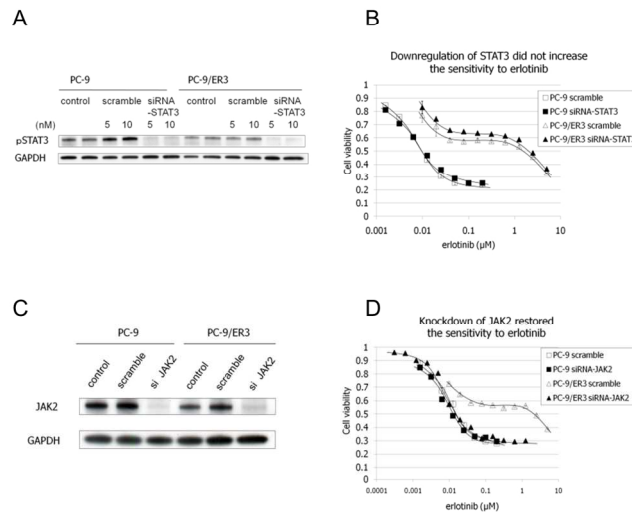
Figure 5



Protein expression in PC-9 and PC-9/ER3 cells upon treatment with erlotinib plus or minus JSI-124. Extracts from PC-9 and PC-9/ER3 cell lines, treated with erlotinib (0.01 μM) or JSI-124 (10 μM) or both for 6 hours, were subjected to Western blotting. Treatment with erlotinib alone inhibited pMAPK in both cell lines and pAkt in PC-9, but not PC-9/ER3, cells. Treatment with JSI-124 alone inhibited pJAK2, pSTAT3, and pMAPK in both cell lines and pAkt in PC-9/ER3, but not in PC-9, cells. pAkt in PC-9 cells treated with both erlotinib and JSI-124 was similar to that with erlotinib alone. However, in PC-9/ER3 cells, JSI-124 moderately suppressed pAkt. The combination of both drugs suppressed pAkt as much as that in PC-9 cells treated with erlotinib alone.

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Figure 6

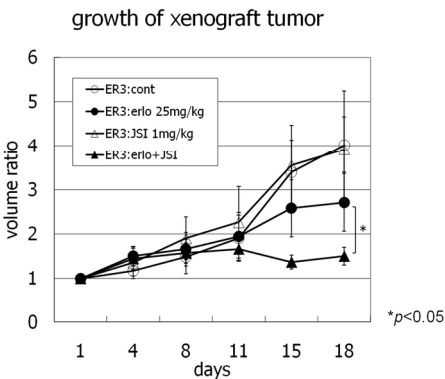


siRNA knockdown of STAT3 and JAK2.

A, STAT3 was knocked down in PC-9 and PC-9/ER3 cells by STAT3-specific siRNA. B, Cells ( $3 \times 10^3$  per well) were seeded in 96-well plates in quadruplicate and grown with the indicated concentration of erlotinib. After 96 hours, the cells were subjected to MTT assays. The knockdown of STAT3 did not affect the sensitivity to erlotinib of either cell line. C, JAK2-specific siRNA lowered JAK2 expression. D, The knockdown of JAK2 did not influence the sensitivity of PC-9 cells to erlotinib, but restored the sensitivity of PC-9/ER3 cells to erlotinib.

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Figure 7



Growth curves of PC-9/ER3 xenograft tumors.  
PC-9/ER3 xenograft model mice were treated with JSI-124 (1 mg/kg/day) alone, erlotinib (25 mg/kg/day) alone, erlotinib in combination with JSI-124, or vehicle alone. JSI-124 alone did not inhibit tumor growth. Erlotinib alone did not significantly affect the tumor volume. The two-drug combination resulted in significant tumor shrinkage compared to either drug alone ( $P < 0.05$ ). Differences in tumor volume were compared using Student's t-test. Bars, standard error.  
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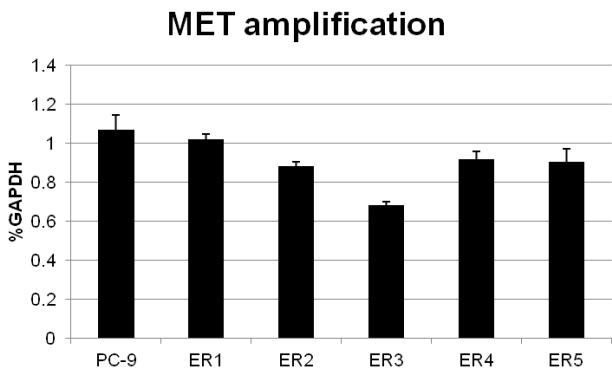
# Supplemental Fig. 1

IC <sub>50</sub> values of erlotinib and gefitinib in PC-9 and PC-9/ER3, 4 and 5 cells determined by MTT assay		
	IC <sub>50</sub> ± SD (μ M)	
	erlotinib	gefitinib
PC-9	0.0089 ± 0.000 *	0.011 ± 0.001 *
PC-9/ER3	1.21 ± 0.11 *	2.78 ± 0.42 *
PC-9/ER4	8.59 ± 3.82 *	7.01 ± 1.08 *
PC-9/ER5	3.30 ± 0.37 *	2.92 ± 1.80 *
*P< 0.05 (t test).		

All the resistant cells were significantly resistant to erlotinib and gefitinib.

# Supplemental Fig. 2

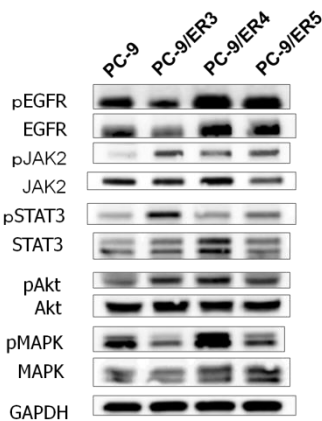
*MET* gene copy number relative to *GAPDH* in PC-9 and PC-9/ER1, 2, 3, 4 and 5 cells.



*MET* gene copy number was examined by quantitative PCR using genomic DNA in three independent experiments. Bars, standard deviation.

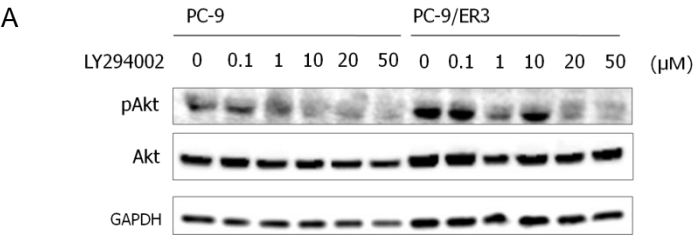
# Supplemental Fig. 3

Protein expression in PC-9 and PC-9/ER3, 4 and 5 cells treated with or without erlotinib for 6 hours.

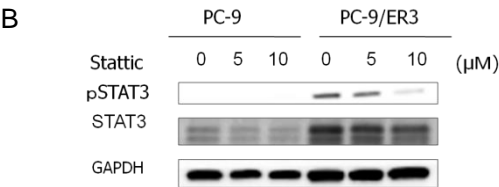


# Supplemental Fig. 4

Target protein expression in PC-9 and PC-9/ER3 cells treated with each indicated concentrations of LY294002 or static.



pAkt was inhibited by LY294002 in cells incubated for 6 hours in a dose-dependent manner.

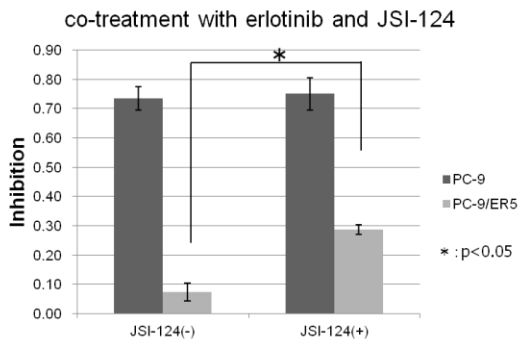


pSTAT3 was inhibited by stattic in cells incubated for 12 hours in a dose-dependent manner.



# Supplemental Fig. 5

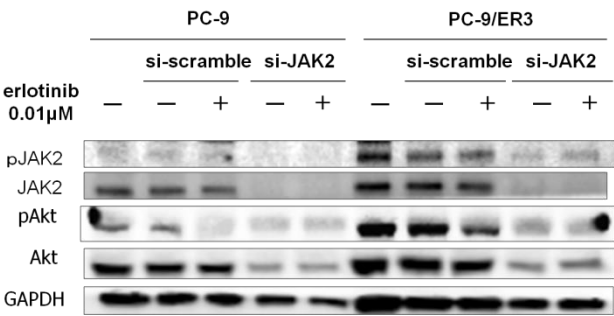
Growth inhibition ratio after treatment with erlotinib plus JSI-124 of PC-9/ER5 cells.



Cells were treated with 10 nM erlotinib plus or minus 30 nM JSI-124 for 96 hours. Growth inhibition relative to erlotinib-untreated cells with or without JSI-124 was shown. Data are representative of three independent experiments. Bars, standard error.

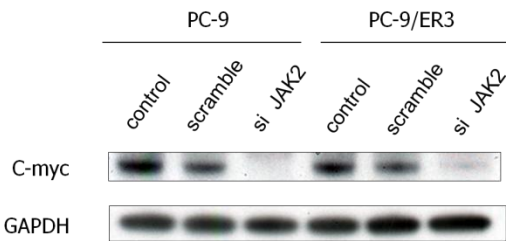
# Supplemental Fig. 6

The combination of erlotinib with inhibition of JAK2 using siRNA in PC-9/ER3 cells suppressed pAkt in PC-9/ER3 cells.

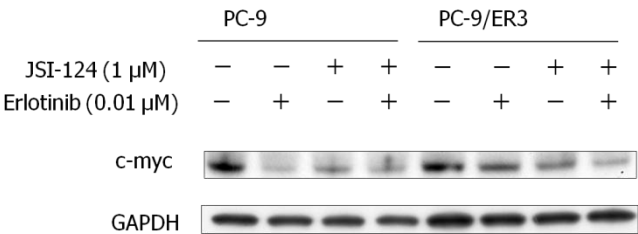


# Supplemental Fig. 7

Inhibition of JAK2 suppresses c-MYC expression.



Western blotting shows that siRNA knockdown of JAK2 inhibited c-MYC protein expression in PC-9 and PC-9/ER3 cells.



Extracts from PC-9 and PC-9/ER3 cell lines, which were treated with erlotinib (0.01  $\mu$ M) or JSI-124 (1  $\mu$ M), or both, for 12 hours, were subjected to Western blotting. The combination of erlotinib and JSI-124 efficiently suppressed c-MYC expression in the PC-9/ER3 cells.