Integrin$\beta_1$ contributes to EGFR-TKI resistance in NSCLC

Erlotinib resistance in lung cancer cells mediated by integrin$\beta_1$/Src/Akt-driven bypass signaling

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**Running title:** Integrin$\beta_1$ contributes to EGFR-TKI resistance in NSCLC
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Conflicts of Interest

The authors have no potential conflicts of interest.
Abstract

EGFR kinase inhibitors including gefitinib and erlotinib exert potent therapeutic efficacy in non-small cell lung cancers harboring EGFR activating mutations. However, most patients ultimately develop resistance to these drugs. Here we report a novel mechanism of acquired resistance to EGFR tyrosine kinase inhibitors and the reversal of which could improve clinical outcomes. In erlotinib-resistant lung cancer cells harboring activating EGFR mutations that we established, there was increased expression of Src, integrinβ1, α2, and α5 along with enhanced cell adhesion activity. Interestingly, RNAi-mediated silencing of integrinβ1 restored erlotinib sensitivity and reduced activation of Src and Akt after erlotinib treatment. Further, Src silencing inhibited Akt phosphorylation and cell growth, with this inhibitory effect further augmented by erlotinib treatment. Increased expression of integrinβ1, α5, and/or α2 was also observed in refractory tumor samples from lung cancer patients treated with erlotinib and/or gefitinib. Together, our findings identify the integrinβ1/Src/Akt signaling pathway as a key mediator of acquired resistance to EGFR-targeted anticancer drugs. (158 words)
Acquired resistance to cancer cell-targeted therapies invariably pose clinical problems for resolution, due to the inherent heterogeneity and plasticity of all human tumors, but combining agents that anticipate common resistance pathways it may be possible to delay relapses.
Introduction

Patients with non-small cell lung cancer (NSCLC) harboring activating somatic mutations in the epidermal growth factor receptor (EGFR) gene show dramatic clinical responses. Of the somatic mutations, in-frame deletions in exon 19 (del E746-A750) and the L858R point mutation are most commonly observed in NSCLC [1]. These EGFR mutations are closely associated with sensitivity to EGFR tyrosine kinase inhibitors (TKIs), erlotinib and gefitinib. Both gefitinib and erlotinib have shown to improve progression-free survival as compared to chemotherapy alone when given as first-line treatment for patients with NSCLCs harboring activating EGFR mutations [2-6].

However, one of the serious problems encountered during clinical treatment with EGFR-TKI is the appearance of drug-resistant tumors [7]. Well-characterized mechanisms for the acquired resistance to EGFR-TKIs include the T790M mutation in exon 20 of the EGFR-TK domain [8, 9] and Met amplification [10]. Furthermore, the loss of PTEN and increased expression of MAPK, ABCG2, IGF1R, and TGF−β have also been reported [7, 11, 12]. In addition to these well-characterized mechanisms, further elucidation of novel mechanisms for acquired drug resistance is essential for the development of personalized therapeutics and strategies to circumvent drug resistance.

In our laboratory, we have established various drug-resistant cell lines from human
lungs cancer cell lines harboring activating EGFR mutations by selecting for resistance to EGFR-TKIs [13]. Previously we have reported the loss of PTEN expression with the loss of nuclear translocation of EGR-1, a transcription factor responsible for PTEN gene expression, in gefitinib resistant clones [14-16]. We also reported that either the complete or partial loss of the activated EGFR gene allele could also result in the acquisition of erlotinib resistance [17]. In this present study, we further established erlotinib-resistant clones by step-wise selection following exposure to erlotinib and observed enhanced expression and activation of integrinβ1 and Src. Herein, we present a novel bypass mechanism through which the integrinβ1/Src/Akt signaling may play a pivotal role in the acquisition of erlotinib resistance in lung cancer cells.
Materials and Methods

Cell culture

PC9 cells were kindly provided by Dr. Y Ichinose, National Hospital Organization, Kyushu Cancer Center, Japan, and 11-18 cells are provided by Dr. K Nakagawa, Kinki University, Japan. These cells and the resistant clones were cultured in RPMI medium supplemented with 10% fetal bovine serum in an atmosphere of 5% CO₂ [14, 18]. Cells were routinely confirmed to be free of mycoplasma contamination using myco sensor QPCR Assay kits (Agilent Technologies). Cells were transiently transfected with cDNA using Lipofectamine LTX, PLus reagent and Opti-MEM medium (Invitrogen) according to the manufacturer’s recommendations. Cells were transfected with small interfering RNA (siRNA) duplexes using Lipofectamine RNAiMAX and Opti-MEM medium (Invitrogen) according to the manufacturer’s recommendations. Reagents are described in Supplementary material and methods.

Cytotoxicity assays

Exponentially growing cell suspensions were seeded into each well and the following day the indicated concentration of drugs were added. After incubation for 72 hr, cytotoxicity was determined as described previously [17, 18]. Each value represents the average from triplicate wells ± SD.
For colony formation assay, $5 \times 10^2$ cells were plated in 35-mm dishes, and following day, cells were transfected with integrin$\beta_1$ siRNA. After 48hr, various concentrations of erlotinib were added, followed by incubation for 7 days at 37 C°. Colonies over 10 cells were counted after Giemsa staining, as described previously [19]. Each value represents the average of duplicate dishes.

**Western blot analysis**

Cells were rinsed with ice-cold PBS and lysed in Triton X-100 buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 50 mmol/L NaF, 1% Triton X-100, and 10% glycerol containing 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 $\mu$g/mL aprotinin, 10 $\mu$g/mL leupeptin, and 1 mmol/L sodium orthovanadate), and cell lysates were separated by SDS-PAGE and transferred to Immobilon membranes (Millipore Corp.). For detection of active integrin$\beta_1$, cells were plated on fibronectin- or collagen-coated dishes, and incubated with anti-active integrin$\beta_1$ antibody (HUTS-4) for 30 min at 37 C°. Cells were then rinsed with PBS, lysed in SDS sample buffer, and bound antibody was detected by western blotting [20]. Immunoprecipitation (IP) was described in Supplementary Material and Methods.

**Cell adhesion assay**

For cell adhesion assay, 96 well plates were coated with collagen (10$\mu$g/well,
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Trevigen) or fibronectin (5µg/well, Trevigen), and cancer cells were seeded onto the ECM components, allowed to adhere for 30 min at 37°C. Each value represents the average from triplicate trials ± SD.

**Quantitative real-time PCR**

RNA was reverse transcribed from random hexamers using avian myeloblastosis virus reverse transcriptase (Promega). In brief, 20 µl of PCR amplification reaction mixtures contained cDNA, primer pairs (Applied Biosystems), a dual-labeled fluorogenic probe (Applied Biosystems), and Taqman Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reactions at 50°C for 2 min and 95°C for 10 min, and then 40 cycles alternating between 95°C for 15 seconds and 60°C for 1 min. The relative gene expression for each sample was determined using the formula $2^{(-\Delta Ct)} = 2^{[Ct \text{ (GAPDH)} - Ct \text{ (target)}]}$, which reflected the target gene expression normalized to glyceraldehyde-3- phosphate dehydrogenase (GAPDH) levels. Each value represents the average from triplicate trials ± SD.

**Flow cytometry (FACS)**

Cell suspension (10⁶ cells/100µl) in incubation buffer (0.5% BSA in PBS) was stained with anti-integirn β1 mAb or Purified PE-Mouse IgG2a Control (Becton-Dickinson) for 1 hr at 4°C. Flow cytometry was performed using the FACS.
Caliber System (Becton-Dickinson).

Tumor samples

Informed consent for the use of tumor tissue specimens was obtained from patients according to the legal guidance [21, 22]. Tumor samples, Case 4 and Case 10, are collected from University Hospital, University of Occupational and Environmental Health, Japan. Tumor samples, Case RB4 and Case RB11, are from EURTAC Study in Spain. The EURTAC was approved by the institutional review board of each participating center, and written informed consent was obtained from all patients. Mutation status of EGFR (delE746-A750 and L858R) and T790M was examined by direct sequencing analysis. K-Ras mutations were investigated by PCR-analysis [21, 22, 23].

Immunohistochemical analysis

Expression of integrins and Src was examined by IHC staining previously described methods [23]. Anti-integrinβ1 mAb was purchased from Abcam Inc., anti-integrinα2 mAb was from Epitomics, anti-integrinα5 mAb was from Santa Cruz Biotechnology and anti-Src was from Cell Signaling Technology. The intensity of staining was scored using the following scale: no staining, 0; weak staining, +1; moderate staining, +2; and strong staining, +3, in >10% of cancer cells.
Results

Establishment of the erlotinib-resistant subclones from PC9 cells and 11-18 cells

To isolate erlotinib-resistant subclones from PC9 cells harboring the activating EGFR delE746-A750 mutation, and 11-18 cells harboring activating EGFR L858R mutation, we cultured PC9 cells and 11-18 cells in increasing, step-wise doses of erlotinib up to 15 μM for approximately 6 months as described previously [14, 17]. We isolated a drug-resistant cell lines, PC9/ER2 and 11-18/ER2, and further established drug-resistant subclones, PC9/ER2-2 and PC9/ER2-3 from PC9/ER2 and 11-18/ER2-1 from 11-18/ER2 (Figure 1A, Table 1). Growth rates between each parental cell line and resistant subclones are similar (Table 1). Resistant subclones also manifested increased resistance to gefitinib as compared to the parental cell lines (Table 1). In contrast, these three resistant subclones exhibited similar sensitivities to picropodophyllin (an inhibitor of IGF1R), SU11274 (an inhibitor of Met), and cisplatin as compared to the parental cell lines (Table 1). Neither alteration for secondary EGFR mutations (T790M, T854A, L747S and D761Y) nor hot spot activating mutation of PIK3CA was detected in resistant subclones (data not shown).

As compared to parental cell lines, all resistant subclones expressed relatively lower levels of phospho-HER2 (pHER2) (Figure 1B). In addition, two resistant subclones from PC9 expressed lower levels of HER2, HER3, and phospho-HER3 (pHER3). In
contrast, the expression of phospho-Akt (pAkt) was moderately higher in the resistant subclones, although that of PTEN, c-Met, IGF1R, Axl and FGFRs was also similar among cell lines (Figure 1B, data not shown). DelE746-A750 EGFR was similarly expressed among both of the resistant subclones and the PC9 line (data not shown). However, L858R EGFR expression was lower in 11-18/ER2-1 cells because of partial loss of mutated EGFR gene allele, as described previously [17]. The phosphorylation of EGFR (pEGFR) and Erk1/2 (pErk1/2) was similarly inhibited by erlotinib in a dose-dependent manner in parental cell lines as well as the resistant subclones. In contrast, the phosphorylation of Akt was observed even in the presence of erlotinib in the resistant subclones (Figure 1C).

**Increased expression of β1, α2, and α5 integrins in the erlotinib-resistant subclones**

We further examined the expression levels of integrins in the resistant subclones by western blot analysis. Surprisingly, all resistant subclones manifested higher levels of β1, α2, and α5 integrins than the parental cell lines. However, there were similar expression levels of αv and β4 integrins between parental cell lines and the resistant subclones (Figure 2A). PC9/ER2-2 and PC9/ER2-3 also exhibited higher expression levels of integrinβ1 mRNA than the PC9 cells, but 11-18/ER2-1 exhibited similar expression levels of integrinβ1 mRNA (Figure 2B). FACS analysis also revealed higher expression
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levels of membranous integrinβ1 in PC9/ER2-2 and 11-18/ER2-1 as compared to parental cell lines (Figure 2C). Integrinβ1 is known to dimerized with α integrin family proteins α2 and α5 [24], and IP western blot analysis revealed that α2 or α5 integrins were co-immunoprecipitated with integrinβ1 in PC9/ER2-2 and PC9/ER2-3 cells (Supplementary Figure S1A).

Integrins induce the activation of downstream regulatory signaling pathways by adopting a high-affinity state to interact with extracellular matrices [25]. Ligand of integrinα2β1 and integrinα5β1 are collagen and fibronectin, respectively. As shown in Figure 2D, increased activation of integrinβ1 in PC9/ER2-2 and 11-18/ER2-1 cells as compared to parental cell lines was observed in the presence of fibronectin or collagen when a specific antibody (HUTS-4) recognizing ligand-occupied integrinβ1 was used. Furthermore, the adhesion of cells to collagen- or fibronectin-coated plates in the resistant subclones was significantly higher than that observed in parental cell lines (Figure 2E).

Integrinβ1 knockdown overcomes erlotinib resistance in resistant subclones

We next examined whether integrinβ1 expression was correlated with erlotinib resistance or constitutive Akt phosphorylation. Treatment with integrinβ1 siRNA almost completely suppressed the expression of integrinβ1 protein and mRNA (Figure 3A, B),
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and there were no apparent changes in the growth rates between each parental cell line and their resistant subclones upon exposure to integrinβ1 siRNA for five days (data not shown). In resistant subclones, Integrinβ1 siRNA-treatment restored sensitivity to erlotinib (Figure 3C), and erlotinib treatment almost completely blocked the constitutive phosphorylation of Akt when integrinβ1 was silenced (Figure 3D). We further examined whether integrins α2 and α5 were also involved in the constitutive Akt phosphorylation in PC9/ER2-2 cells (Supplementary Figure S1B). The phosphorylation of Akt was moderately inhibited by erlotinib when integrinα2 or integrinα5 was silenced in PC9/ER2-2 cells. Integrinβ1 knockdown was also accompanied by the downregulation of integrinα5, suggesting a close regulatory link between integrinβ1 and integrinα5. These results suggest that integrinβ1, in concert with integrinα2 and/or integrinα5, appears to play a key role in the acquisition of erlotinib resistance.

We further established the stable cell lines PC9/ITG2 and PC9/ITG11 by transfecting PC9 cells with integrinβ1 cDNA (Supplementary Figure S2A). There was no concomitant enhancement of the expression of integrinα2 or integrinα5 in either cell line, and no apparent changes in the growth rates of PC9/Mock, PC9/ITG2 and PC9/ITG11 cells over the 5-day culture period (data not shown). These two stable cell lines exhibited slightly increased resistance to erlotinib (Supplementary Figure S2B). Furthermore, the
phosphorylation of Akt was not suppressed by erlotinib in either the PC9/ITG2 or PC9/ITG11 cell lines, whereas Akt phosphorylation was suppressed in PC9/Mock cells (Supplementary Figure S2C). The phosphorylation of EGFR and Erk1/2 was similarly suppressed by erlotinib in the PC9/Mock cells and the corresponding integrinβ1-overexpressing cell lines. Thus, the overexpression of integrinβ1 with integrinα2 and integrinα5 appears to be one of the mechanisms underlying acquired erlotinib resistance.

**Src knockdown and dasatinib treatment suppress Akt phosphorylation and cell survival in the resistant subclones**

Src protein, which interacts with PI3K, is often activated by EGFR or integrinβ1 in association with extracellular matrices [26]. Thus, we next examined whether integrinβ1 could directly induce the activation of the Src/Akt pathway and whether this pathway could be responsible for erlotinib resistance. Relatively higher expression levels of Src and pSrc (Y416) were observed in PC9/ER2-2 and 11-18/ER2-1 cells as compared to each parental cell line (Figure 4A). In each resistant subclone from PC9 or 11-18 cells, the phosphorylation of Src was blocked by integrinβ1 knockdown and this reduction was augmented by erlotinib (Figure 4B), suggesting a close correlation between integrinβ1 and
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EGFR with the activation of the Src/Akt pathway.

We next examined how Src activation could be involved in the integrin β1-driven activation of Akt and acquired erlotinib resistance using Src siRNA and dasatinib, a Src kinase inhibitor. Treatment with Src siRNA almost completely suppressed the expression of Src (Figure 4C). Src siRNA treatment suppressed expression of Src and markedly suppressed Akt phosphorylation (Figure 4D) and cell growth (Figure 4E) in PC9/ER2-2 and 11-18/ER2-1 cells. This suppression of Akt phosphorylation was augmented by erlotinib. We further examined whether Akt phosphorylation was inhibited by erlotinib in PC9/ER2-2 cells treated with an inhibitor of Src, dasatinib. Dasatinib treatment at 0.1 μM suppressed Akt phosphorylation, and this effect was augmented by erlotinib in PC9/ER2-2 cells (Supplementary Figure S3A). Moreover, PC9/ER2-2 cells were more sensitive to dasatinib than PC9 cells (Supplementary Figure S3B), suggesting that Src activation in the resistant subclone may sensitize those cells to the cytotoxic effects of dasatinib.

The expression of integrins in cancer cells from refractory lung tumors

We finally examined whether integrin expression could be observed in gefitinib- or erlotinib-refractory tumors. Four clinical samples were prepared from lung adenocarcinoma patients treated with gefitinib or/and erlotinib, but subsequently became...
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refractory to these EGFR-TKIs (Supplementary Table S1) [21]. As shown in Supplementary Table S1, two patients harbored L858R and two patients harbored delE746-A750. Figure 5 shows typical immunohistochemistry images from the patients. Especially expression of integrinβ1 was increased in the post-erlotinib and post-gefitinib tumor samples with enhanced expression of integrinα2 or α5. Src expression was also enhanced after treatment, but only post-erlotinib sample of Case 10 shows lower expression of Src than pre-treatment sample. One sample, Case RB4 harboring T790M secondary mutation of EGFR, shows higher expression of integrinβ1, α2 and Src in post-gefitinib treatment. In this case, integrins overexpression might not be directory correlated with acquired resistance (Figure 5D). Expression of integrins and Src in four clinical samples was summarized in Supplementary Table S1.
Discussion

In our present study, we established erlotinib-resistant subclones from PC9 cells harboring the activating del E746-A750 EGFR mutation and 11-18 cells harboring the activating L858R EGFR mutation. We observed the following characteristics in these resistant subclones: 1) Akt was constitutively activated, and the phosphorylation of Akt was not suppressed by erlotinib in resistant subclones; 2) the expression of β1, α2 and α5 integrins was upregulated along with Src activation; 3) the knockdown of integrinβ1 restored cellular sensitivity to erlotinib and reduced Akt phosphorylation; 4) Src knockdown or dasatinib treatment effectively inhibited cell survival and reduced Akt phosphorylation in the presence or absence of erlotinib; and 5) enhanced expression of integrin β1, α2 and/or α5 was also observed in refractory tumor samples from lung cancer patients treated with gefitinib and/or erlotinib. Together, these novel findings indicate that the acquisition of erlotinib resistance is mediated by the activation of the integrinβ1/Src/Akt signaling pathway (Figure 6).

Three major mechanisms including the alteration of oncogenic targets, the activation of downstream regulatory molecules, and the activation of bypass effectors, are known to contribute to acquired resistance to molecularly targeted drugs in cancer cells [7]. The present study suggests that the acquisition of erlotinib resistance is attributable to the
activation of an integrinβ1-driven bypass signaling pathway. Furthermore, the study by Ju et al. [27] was previously reported that transfection with integrinβ1 cDNA protected PC9 cells from apoptosis in response to gefitinib treatment. However, this study did not evaluate how integrinβ1 overexpression renders lung cancer cells resistant to EGFR-TKIs. Our study demonstrated that transfection with integrinβ1 slightly induced erlotinib resistance in PC9 cells (Supplementary Figure S2), and in these integrinβ1-overexpressing cell lines, there was no enhanced expression of integrinα2 or α5, suggesting that the co-activation of β1/α2 and/or β1/α5 integrins is required for the acquisition of increased erlotinib resistance via the integrinβ1 signaling pathway.

The interactions between cancer cells and the extracellular matrices often reduce the sensitivity to anticancer agents, including gefitinib [28-31], and the expression of integrin family proteins plays an important role in the malignant progression of cancers [32-34]. The cell proliferation rates were similar between parental cell lines and the resistant subclones, but the adhesion to collagen or fibronectin was much higher in the resistant subclones. Integrinβ1 thus appears to be responsible for not only the acquisition of erlotinib resistance but also for cell adhesion activity.

In the current study, we observed constitutive phosphorylation of Akt even in the presence of erlotinib in resistant subclones. This phosphorylation was markedly
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suppressed by erlotinib upon integrinβ1 knockdown, and cell sensitivity to erlotinib was restored by integrinβ1 knockdown. These data strongly suggest an essential role for integrinβ1/α2 and/or integrinβ1/α5 in the constitutive activation of Akt and in the drug resistance in the resistant subclones. Our study also sought to understand how integrinβ1 promotes Akt phosphorylation and the acquisition of drug resistance. Of the potentially relevant molecules, Src is known to be a downstream regulator of EGFR and/or integrins, and it plays an essential role in the survival of lung cancer cells [35, 36]. Src is also responsible for the acquisition of resistance to EGFR-targeted drugs [37, 38]. Consistent with these studies, we observed marked activation of Src in PC9/ER2-2 and 11-18/ER2-1 cells as compared to the parental counterparts. The blockade of Src signaling by Src siRNA resulted in the marked suppression of Akt phosphorylation, suggesting a close link between Src and Akt activation, as demonstrated in a previous study [26]. Furthermore, Src knockdown also dramatically suppressed cell growth in PC9/ER2-2 and 11-18/ER2-1 cells even in the absence of erlotinib. Furthermore, treatment with a Src kinase inhibitor dasatinib also suppressed Akt phosphorylation. Taken together, these data suggest that the activation of the integrinβ1/Src/Akt signaling pathway may undergo compensatory activation during the acquisition of drug resistance (Figure 6). On the other hand, clinical trials of Src inhibitor dasatinib in combination with erlotinib was studied in lung cancer...
Integrin $\beta_1$ contributes to EGFR-TKI resistance in NSCLC [39]. We suggest that Src is one of the effective target for lung cancer therapeutics.

We also examined whether integrin $\beta_1$, together with integrin$\alpha_2$ or integrin$\alpha_5$, was expressed in lung cancer refractory to gefitinib and/or erlotinib. These results demonstrated increased expression of $\beta_1$, $\alpha_2$ and/or $\alpha_5$ integrins in four refractory tumors following treatment with gefitinib and/or erlotinib (Figure 5, and Supplementary Figure S1). Enhanced expression of integrin$\beta_1$ and $\alpha_2$ were also observed in case RB4 harbored T790M secondary EGFR mutation (Figure 5D). It remains to be further studied whether the enhanced expression of integrin$\beta_1$, $\alpha_2$ and Src is closely correlated with T790M mutation. Recently, integrin family targeted drugs are focused on cancer therapeutics. For example, anti-integrin$\alpha_5\beta_1$ antibody (volocixmab) is performed on phase II clinical trials for several cancers including NSCLC [40, 41]. Further study should be also required whether these integrin-targeted drugs could be useful to overcome drug resistance in patients with NSCLC.

In conclusion, the acquisition of erlotinib resistance in our erlotinib-resistant subclones was attributable to the overexpression of integrin$\beta_1/\alpha_2/\alpha_5$, which resulted in the activation of an integrin/Src/Akt pathway. This relationship between integrin expression and Src activity may be useful for the development of both personalized therapeutics involving EGFR-TKIs and for circumventing drug resistance in patients with NSCLC.
Grant Support

This work was supported by the 3rd Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare in Japan and also by a Grant-in-Aid for challenging Exploratory Research from the Japan Society for the Promotion of Science (JSPS) (M. Ku.).
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Acquired resistance to gefitinib: the contribution of mechanisms other than the T790M, MET, and HGF status. *Lung Cancer* 2010;68:198-203.


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Table 1. Comparison of drug sensitivity to various molecular targeted drugs and cisplatin

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Doubling time(hr)b</th>
<th>Relative drug resistance a</th>
<th>Erlotinib</th>
<th>Gefitinib</th>
<th>Picropodophyllin</th>
<th>SU11274</th>
<th>cisplatin</th>
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<td>PC9</td>
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<td>1.0(0.029)</td>
<td>1.0(0.011)</td>
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<td>331(3.9)</td>
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<td>1.1(5.0)</td>
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<td>PC9/ER2-3</td>
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<td>310(3.6)</td>
<td>0.77(0.4)</td>
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<tr>
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aIC₅₀ values (μmol/l) are calculated from logit regression lines from triplicate dishes, and presented in parenthesis. The relative drug resistance is defined as the IC₅₀ value divided by the value for each parental cell line.

bDoubling time (hr) for each cell lines is average of duplicate trials.
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Figure and table legends

Table 1. Comparison of drug sensitivity to various molecular targeted drugs and cisplatin.

Figure 1. Expression and activation of receptor tyrosine kinases and downstream signaling molecules in PC9, PC9/ER2-2, PC9/ER2-3, 11-18, and 11-18/ER2-1 cells. A, The sensitivity to erlotinib was assessed by cell proliferation assays for 3 days. B, Western blots showing the expression levels of proteins, and the loading control α-tubulin. C, Western blots showing the effect of erlotinib on expression of proteins. Each cell line was exposed to various doses of erlotinib for 5 hr.

Figure 2. Increased expression and activation of Integrinβ1 in erlotinib-resistant subclones. A, Western blots showing the expression levels of integrins and α-tubulin as a loading control. B, Integrinβ1 mRNA levels were determined by qRT-PCR. *P < 0.05, **P < 0.01 versus each parental cell line. C, Flow cytometry of the cell surface expression of integrinβ1. D, Western blots showing the expression level of active integrinβ1 using the HUTS-4 antibody, which recognizes ligand-occupied integrinβ1. Plastic dishes were
Integrin \( \beta_1 \) contributes to EGFR-TKI resistance in NSCLC coated with fibronectin (FN), or collagen (CL). E, Cell adhesion assay on plastic dishes coated with BSA, collagen, or fibronectin. \( *P < 0.05, **P < 0.01 \) versus each parental cell line.

**Figure 3. Effect of integrin\( \beta_1 \) knockdown on drug sensitivity in erlotinib-resistant cell lines.** A, Western blot showing integrin\( \beta_1 \) levels after treatment with siRNA for the indicated number of days. B, Integrin\( \beta_1 \) mRNA expression was analyzed by qRT-PCR after 2 days of siRNA treatment. \( *P < 0.05, **P < 0.01 \) versus each parental cell line. C, The effect of integrin\( \beta_1 \) knockdown on the sensitivity to erlotinib treatment for 7 days was assessed by the colony formation assay. D, The effect of integrin\( \beta_1 \) knockdown on the phosphorylation of EGFR, Akt, and Erk1/2. The cells were treated with siRNA for 2 days followed by exposures to 1 \( \mu \)M erlotinib for 5 hr.

**Figure 4. Effect of Src knockdown on Akt phosphorylation and the restoration of erlotinib sensitivity by Src knockdown.** A, Western blots demonstrating the expression of p-Src (Y416) and Src. B, The effect of integrin\( \beta_1 \) knockdown on Src phosphorylation with or without erlotinib. Cells were treated for 2 days with integrin\( \beta_1 \) siRNA, followed by further exposure to erlotinib for 5 hr. C, Western blots demonstrate the expression of
Src protein after treatment with Src siRNA for the indicated number of days. Because Src and α-tubulin were detected on the same membrane, α-tubulin is pointed with arrowheads.

D, The cells were treated with or without Src siRNA for 2 days, exposed to erlotinib for another 5 hr. The quantitative analysis of the western blots is shown, and the values are normalized to pAkt levels in the absence of erlotinib and Src siRNA. E, The effects of Src knockdown on proliferation of cells. Cells were treated for 2 days with Src siRNA, followed by treatment with erlotinib. The cell number in the absence of the drug and/or siRNA was set as 100%.

Figure 5. Immunohistochemical analysis of clinical tumor samples.

Immunohistochemical analysis of integrins or Src expression in patients refractory to gefitinib and/or erlotinib treatment. EGFR mutation status of each tumor sample was shown at the left side. Magnification, ×200. A, B, Tumor samples were derived from Japan, and C, D, from Spain.

Figure 6. Our hypothetical model how erlotinib resistance is acquired in lung cancer cells. In erlotinib sensitive cell lines, the proliferation and survival of human lung cancer cells harboring activating EGFR mutation depend upon the EGFR-driven PI3K/Akt
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pathway, and this cell proliferation and survival is highly susceptible to erlotinib and other EGFR-TKIs. Erlotinib-resistant subclones express elevated levels of β1 and α2/α5 integrins together with Src, resulting in Akt activation. In erlotinib resistant cell lines, activated integrinβ1, α2 or α5 and Src bypass pathway contributes to Akt signaling pathway.
Figure 1

A

B

C

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Figure 3

A

B

C

D

PC9

PC9/ER2-2

PC9/ER2-3

Erlotinib (μM)

Erlotinib (μM)

Erlotinib (μM)
Figure 4

A

B

C

D

E

Figure 4
**Figure 5**

A. Case 4 (L858R)

Pre-Gefitinib

Post-Gefitinib

B. Case 10 (L858R)

Pre-Gefitinib

Post-Erlotinib

C. Case RB11 (ΔE476-A750)

Pre-Erlotinib

Post-Erlotinib

D. Case RB4 (ΔE746-A750, T90M)

Pre-Gefitinib

Post-Gefitinib
Figure 6

Acquired resistance to Erlotinib

EGFR/EGFR family

Inactive integrins

EGFR/EGFR family

Active integrins

Mutant EGFR

Wild EGFR

EGFR family

AKT

Cell survival

Src

AKT

Cell survival
Erlotinib resistance in lung cancer cells mediated by integrin β1/Src/Akt-driven bypass signaling

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