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Slug Confers Resistance to the Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor

Tzu-Hua Chang[1], Meng-Feng Tsai[2], Kang-Yi Su[3,4], Shang-Gin Wu[5], Cheng-Po Huang[6], Sung-Liang Yu[6,7], Yung-Luen Yu[8], Chou-Chin Lan[1], Chih-Hsin Yang[9], Shwu-Bin Lin[6,7], Chin-Pyng Wu[1,10*], Jin-Yuan Shih[11*], and Pan-Chyr Yang[1,4,11*]

Affiliations of Authors: [1]Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan; [2]Department of Molecular Biotechnology, Dayeh University, Changhua, Taiwan; [3]Institute of Statistical Science, Academia Sinica, Taipei, Taiwan; [4]Division of Genomic Medicine, Research Center for Medical Excellence, National Taiwan University, Taipei, Taiwan; [5]Department of Internal Medicine, National Taiwan University Hospital Yun-Lin Branch, Yun-Lin, Taiwan; [6]Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University, Taipei, Taiwan; [7]Department of Laboratory Medicine, National Taiwan University Hospital, Taipei, Taiwan; [8] Graduate Institute of Cancer Biology and Center for Molecular Medicine, China Medical University and Hospital, Taichung, Taiwan; [9]Graduate Institute of Oncology, Cancer Research Center, National Taiwan University; [10]Department of Critical Care Medicine, Li-Shin Hospital, Taoyuan, Taiwan; [11]Department of Internal Medicine, National Taiwan University Hospital,

College of Medicine, National Taiwan University, Taipei, Taiwan

* **These authors contributed equally to this work**

Corresponding Authors:

Chin-Pyng Wu

Graduate Institute of Medical Sciences,

National Defense Medical Center,

#161, Sec. 6, Minquan E. Rd., Neihu Dist., Taipei City 114, Taiwan

Telephone: +886-3-494-1234 ext. 2017

Fax: +886-3-492-1000

E-mail: chesst@ndmctsgh.edu.tw.

Jin-Yuan Shih,

Department of Internal Medicine,

National Taiwan University Hospital,

7 Chung-Shan South Road, Taipei 100, Taiwan.

Telephone: +886-2-2312-3456 ext. 62905

Fax: +886-2-2358-2867

E-mail: jyshih@ntu.edu.tw

or

Pan-Chyr Yang

Department of Internal Medicine,

National Taiwan University Hospital,

7 Chung-Shan South Road, Taipei 100, Taiwan.

Telephone: +886-2-2312-3456 ext. 62905

Fax: +886-2-2358-2867

E-mail: pcyang@ntu.edu.tw

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Running title

Slug confers resistance to EGFR TKI

At a Glance Commentary

Scientific Knowledge on the Subject

Molecular-targeting therapeutics, such as EGFR tyrosine kinase inhibitors, have become an important part of lung cancer treatment strategies. One challenge confronted by targeted cancer therapies is the potential for drug resistance development. Therefore, it is important to understand the molecular mechanisms by which tumors acquire resistance to therapeutic drugs and develop more effective therapeutic interventions.

What This Study Adds to the Field

In this study, we found that expression of the epithelial-mesenchymal transition regulator Slug caused resistance to gefitinib in lung adenocarcinoma. We further discovered that silencing Slug in gefitinib-resistant cells restored gefitinib-induced apoptosis, a process that was mediated mainly through restoration of Bim expression and caspase-9 activation.

This article has an online data supplement, which is accessible from this issue's table of contents online at www.atsjournals.org

Abstract

Rationale: Non-small cell lung cancers carrying epidermal growth factor receptor (EGFR) mutations respond well to EGFR tyrosine kinase inhibitors (TKIs), but patients ultimately develop drug resistance and relapse. Although epithelial-mesenchymal transition (EMT) can predict resistance to EGFR TKIs, the molecular mechanisms are still unknown.

Objectives: To examine the role of EMT regulators in resistance to gefitinib.

Methods: The expression level of EMT regulators in gefitinib-sensitive cells (PC9) and gefitinib-resistant cells (PC9/gef) was determined using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Molecular manipulations (silencing or overexpression) were performed to investigate the effects of EMT regulators on gefitinib resistance in vitro, and a xenograft mouse model was used for in vivo confirmation. In addition, cancer cells from fifty-four patients with malignant pleural effusions of lung adenocarcinoma were collected for analysis of EMT regulator mRNA by quantitative real-time RT-PCR.

Measurements and Main Results: Slug expression, but not that of snail, twist or zeb-1, was significantly increased in PC9/gef compared to PC9 cells. Slug knockdown in PC9/gef cells reversed resistance to gefitinib, and overexpression of Slug in PC9 cells protected cells from gefitinib induced-apoptosis. Silencing of Slug in

gefitinib-resistant cells restored gefitinib-induced apoptosis primarily through Bim upregulation and activation of caspase-9. Slug enhanced tumor growth in a xenograft mouse model, even with gefitinib treatment. In clinical samples, Slug expression was significantly higher in cancer cells with resistance to EGFR TKIs than in treatment-naïve cancer cells.

Conclusions: Slug contributes to the resistance to gefitinib and may be a potential therapeutic target for treating resistance to EGFR TKIs.

Keywords: Slug; gefitinib resistance; apoptosis; epithelial mesenchymal transition

Introduction

Lung cancer is the leading cause of cancer-related death throughout the world. Traditional therapeutic strategies—chemotherapy and radiotherapy (1)—are often associated with unsatisfying outcomes, a problem exacerbated by difficulties of early detection. Molecular-targeted therapy has emerged as an alternative treatment for lung cancer patients, but variable responsiveness rates and the development of drug resistance have created challenges in clinical practice (2). Thus, the development of more effective therapeutic interventions based on the molecular mechanisms by which tumors develop resistance to therapeutic drugs is an urgent need.

Altered expression of the epidermal growth factor receptor (EGFR) has been identified in a variety of human tumors, including lung, breast, head and neck, and ovarian cancers (3-4). Activated EGFR has been reported to promote cell survival, proliferation, invasion, and metastasis through activation of JAK/STAT (Janus kinase/signal transducers and activators of transcription), PI3K (phosphoinositol 3-kinase)/Akt and MAPK (mitogen-activated protein kinase) pathways (4-5). These observations have established EGFR as a target for cancer therapy, and have led to the development of the EGFR tyrosine kinase inhibitors, gefitinib and erlotinib. Recent research has indicated that non-small cell lung cancer (NSCLC) patients with *EGFR*-activating mutations exhibit a dramatic clinical response to EGFR TKIs (6-7).

In clinical practice, Asians, females, non-smokers and patients with adenocarcinoma respond preferentially to EGFR TKIs (8). These are also the patient groups with a high rate of *EGFR* mutations. Despite the dramatic initial responses to such inhibitors, most patients ultimately develop drug resistance and relapse.

Most activating mutations in the *EGFR* gene result in a single substitution of arginine for leucine at position 858 (L858R) and 15-nucleotide deletion (del_E746-A750) in exon 19 (9). Several clinical studies have shown that a second-site point mutation at position 790 (T790M) is responsible for approximate half of all cases of lung adenocarcinoma patients who develop resistance to EGFR TKIs (8, 10). Amplification of the proto-oncogene *MET* (encoding the hepatocyte growth factor receptor) also contributes to EGFR TKI resistance, and is detected in about 20% of these patients (11). The possibility that other mechanisms may also be involved in EGFR TKI resistance cannot be excluded.

Epithelial-mesenchymal transition (EMT), a process by which epithelial cells lose cell polarity and convert to a mesenchymal phenotype, is essential for embryonic development, cancer progression, and chemotherapy resistance (12-14). EMT plays an important role in resistance to EGFR TKIs, during which cancer cells lose epithelial marker, such as E-cadherin (15-17). In contrast, strong expression of E-cadherin enhances gefitinib sensitivity in NSCLC cells with a mesenchymal

phenotype (17). Although EMT can predict resistance to gefitinib or erlotinib (15-16, 18), the molecular mechanisms are still unknown.

Transcriptional reprogramming via the transcription factors Slug, snail, zeb-1, and twist can lead to EMT. These EMT regulators can repress the expression of E-cadherin and have been reported to affect sensitivity to chemotherapeutic drugs (19-20). However, the mechanism of action of EMT regulators with respect to resistance to anticancer drugs, especially EGFR TKIs, is not well defined at present. Here, we found that the EMT regulator Slug contributes to the development of resistance to gefitinib in lung adenocarcinomas containing *EGFR*-activating mutations. Therefore, inhibition of Slug may be an effective strategy for more successful treatment of gefitinib-resistant lung cancers. Some of the results of these studies have been reported in the form of abstract (21).

MATERIALS AND METHODS

Cells and Drugs

The human lung adenocarcinoma cell line PC9 and derivative PC9/gef clones were gifts from Dr. Yang CH (Graduate Institute of Oncology, Cancer Research Center, National Taiwan University). HCC827 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Slug was silenced in PC9/gef cells using small interfering RNA (siRNA) duplexes targeting human Slug (SNAI2), synthesized by Ambion (Austin, TX). Slug overexpression in PC9 cells was accomplished by infecting cells with lentiviruses containing the entire human Slug coding region, prepared using the ViraPower Lentiviral Expression System (Invitrogen, Carlsbad, CA), as described by the manufacturer. A stock solution of gefitinib, a gift from AstraZeneca (London, United Kingdom), was prepared in DMSO and stored at -20°C.

EGFR Mutation Analysis using Sequenom MassARRAY® System

EGFR mutations were detected by MALDI-TOF MS according to the user manual guidelines for the MassARRAY system (SEQUENOM, San Diego, CA) (22).

Cytotoxicity Assay

The colorimetric MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit; Promega, Madison, WI) was used to determine the number of

viable cells. The absorbance at 490 nm was recorded using a VICTOR^3 multilabel reader (PerkinElmer, Waltham, MA).

Immunofluorescence Staining

Cells grown on chamber slides (BD Bioscience, San Diego, CA) were fixed in 4% paraformaldehyde for 15 min. Fixed cells were incubated in blocking solution (0.1% saponin and 0.2% BSA in phosphate-buffered saline [PBS]) to prevent nonspecific staining. The cells were then incubated with primary antibodies for 1 h and then incubated with fluorescence-conjugated secondary antibodies for 30 min. After immunostaining, the chamber slides were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Louis, MO).

Apoptosis Assay

Apoptosis was detected using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). Cells were trypsinized and washed twice with ice-cold PBS. The cell pellet was resuspended in Annexin V binding buffer. FITC-conjugated Annexin V (1 μg/mL) and propidium iodide (50 μg/ mL) were added to the cells and incubated for 15 min at room temperature. Cells were analyzed using a Cytomic FC500 flow cytometer (Beckman Coulter, Brea, CA). The activity of caspase-9 was measured with a luminescence kit (caspase-Glo 9 assays; Promega).

Xenograft Studies in Athymic Mice

Animal experiments were approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taiwan. Four-to-six-week-old nu/nu athymic male mice were obtained from the Laboratory Animal Center of National Applied Research Laboratories (Taipei, Taiwan). Cells were injected subcutaneously into the lower rear flank of the mice. When tumor volumes reached ~200 mm^3, as measured by calipers, mice were randomly allocated into groups of six animals to receive gefitinib (10 mg/kg/day) or vehicle by oral gavage. All mice were sacrificed on day 20 after their tumors had been measured.

Malignant Pleural Fluids Isolation and Culture

This study was approved by the Institutional review board (IRB) of National Taiwan University Hospital (NTUH). Between July 2009 and March 2010, we consecutively collected the pleural effusions from patients who received thoracentesis in the chest ultrasonography examination room of the NTUH. The pleural fluids of patients were acquired aseptically in vacuum bottles by thoracentesis. The red blood cells in the specimen were hemolyzed using RBC lysis buffer. The remaining cells were washed twice with PBS and cultured in complete RPMI1640 media, as described previously (23). Media were replaced every 2–3 days and cells were harvested after 10 days. Total RNA was extracted from cultured cells using the TRIzol reagent (Invitrogen), and the expression of Slug mRNA was determined using

quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), as previously described (24).

Statistical analysis

Student's *t*-test was used to compare the means of two groups. Two-sided *p*-values less than 0.05 were considered significant. All analyses were performed using SPSS software (version 15.0 for Windows; SPSS Inc.) Additional details of measurement techniques are available in an online data supplement.

RESULTS

Gefitinib resistance in PC9/gef cells is not due to EGFR T790M or *MET* amplification.

PC9 cells expressing a mutant *EGFR* with a deletion in exon 19 are a gefitinib-sensitive NSCLC cell line (25). PC9/gef cells were selected from parental PC9 cells that had been continuously exposed to increasing concentrations of gefitinib (Dr. CH Yang; personal communication). PC9 and PC9/gef cells were exposed to gefitinib and cell viability was evaluated using an MTS assay. The viability of PC9/gef cells was unaffected by increasing concentrations of gefitinib up to 5 μM (IC_{50} > 5 μM), whereas PC9 cells were very sensitivity to gefitinib, with an IC_{50}-value of 0.041 μM (Figure 1A). A T790M mutant of *EGFR* and *MET* amplification are the known mechanisms of acquired gefitinib resistance in lung cancer. We analyzed *EGFR* for the DNA substitution corresponding to the T790M mutation by MALDI-TOF MS genotypic analysis and direct sequencing. As shown in Figure 1B and Supplementary Figure E1, we found no evidence for this mutation in parental PC9 cells, PC9/gef cells, or PC9/gef subclones (PC9/gef B4, PC9/gef C2, PC9/gef C4, PC9/gef C7). Quantification of *MET* gene copy number by real-time PCR showed that no *MET* amplification occurred in gefitinib-resistant PC9/gef or subclones (Figure 1C). These results suggest that other mechanisms are responsible for resistance to gefitinib in PC9/gef cells.

The EMT regulator Slug is overexpressed in PC9/gef.

To determine whether EMT contributes to gefitinib resistance in mutant

EGFR-expressing lung cancer cells, we analyzed the expression of the epithelial marker E-cadherin and the mesenchymal marker vimentin by Western blotting and immunofluorescence staining. As shown in Figure 2A, the expression of E-cadherin was decreased in PC9/gef cells compared to that in PC9 cells; in contrast, vimentin was increased in PC9/gef cells. Furthermore, the invasivity of PC9/gef cells was increased relative to that of PC9 cells (Figure 2B). These results indicate that gefitinib-resistant PC9/gef cancer cells had undergone EMT. The transcription factors Slug, snail (SNAI1), zeb-1 and twist can induce EMT (26-27). To verify that resistance to gefitinib was associated with EMT-related transcription factors, we explored the expression levels of snail, Slug, twist, and zeb-1 by Western blotting and quantitative real-time RT-PCR. As shown in Figure 2C, the protein expression of Slug, but not that of snail, twist or zeb-1, was markedly increased in gefitinib-resistant PC9/gef cells. The expression of Slug mRNA in PC9/gef clones was clearly higher than in PC9 cells, and the expression levels of snail, twist, and zeb-1 were not increased in gefitinib-resistance subclones (Figure 2D). Taken together, these data indicate that Slug expression may play a crucial role in gefitinib resistance in this cell model.

Knockdown of Slug reverses gefitinib resistance in PC9/gef cells.

We evaluated the effect of Slug on gefitinib resistance in PC9/gef cells by siRNA-mediated knockdown of Slug. First, we established which siRNA most efficiently knocked down Slug expression in CL1-5 cells, which highly express Slug (Figure 3A),

selecting Slug siRNA 3 (si-Slug 3) over other Slug siRNAs (si-Slug 1, si-Slug 2). Treatment of PC9/gef cells with 50 nM si-Slug 3 (control) for 48 h reduced the levels of Slug and vimentin compared to that in cells treated with scrambled siRNA (si-scramble), but increased E-cadherin levels (Figure 3B and C). Transfection of PC9/gef cells with si-Slug 3 enhanced gefitinib-induced apoptosis, increasing the percentage of apoptotic cells from 12% to 30% (Figure 3D). Knockdown of Slug expression in PC9/gef by a commercial pool of Slug siRNA (pool si-Slug) also increased the percentage of apoptotic cells after gefitinib treatment (Supplementary Figure E2). These results suggest that knockdown of Slug expression reverses gefitinib resistance in PC9/gef cells. Moreover, even in the absence of gefitinib treatment, transfection of si-Slug 3 resulted in about ten percentages of apoptotic cells compared to that of si-scramble, indicating that Slug depletion may induce apoptosis of PC9/gef cells independent of gefitinib treatment. PC9/gef cells transfected with si-Slug 3 or si-scramble and treated with 0.05 μM gefitinib were then assayed for caspase-9 activity by Western blotting and luminescent assays. As shown in Figure 3F and G, gefitinib induced a marked increase in the activity of caspase-9 in PC9/gef cells after knockdown of Slug.

Slug protects PC9 cells from gefitinib-induced apoptosis.

To investigate if Slug plays a protective role against gefitinib-induced apoptosis, we established Slug-overexpressing clones from gefitinib-sensitive PC9 and HCC827 cells, both of which express *EGFR* mutants containing deletions in exon 19. As shown in Figure 4A and

Supplementary Figure E4A, Slug-transfected clones (PC9-Slug and HCC827-Slug) expressed higher levels of Slug protein than the control clones (PC9-mock and HCC827-mock). Moreover, after gefitinib treatment, the percentage of apoptotic cells in cultures of PC9-Slug and HCC827-Slug decreased compared with that in control clones (Figure 4B and C). Similar results were obtained after overexpression of Slug in the SK-MES-1 lung cancer cell line, which is sensitive to gefitinib by virtue of expression of wild-type EGFR (17). In these cells, gefitinib treatment of cells transiently transfected with Slug reduced the percentage of cell death compared to that in mock-transfected controls (Supplementary Figure E3). To investigate whether Slug overexpression affected caspase-9 activities after gefitinib treatment, we used Western blot analysis and caspase activity assays. As shown in Figure 4C and D, after treatment with gefitinib for 48 h, caspase-9 activity was attenuated in Slug-overexpressing cells. These results indicate that high levels of Slug may provide protection against gefitinib-induced apoptosis. Next, to determine whether overexpression of Slug protected PC9 cells against gefitinib treatment in vivo, we inoculated athymic nude mice with PC9-Slug or PC9-mock cells and then administered 10 mg/kg/day gefitinib or vehicle control. As shown in Figure 4E, the administration of gefitinib induced significant regression of PC9-mock tumor xenografts, but not of PC9-Slug xenografts, compared with vehicle groups ($p = 0.003$).

Slug suppresses Bim to inhibit gefitinib-induced apoptosis.

In previous reports, Slug has been shown to protect cells through regulation of

anti-apoptotic (28) and BH3-only proteins (29). Our results showed that gefitinib-induced apoptosis in Slug-silenced PC9/gef cells was associated with the intrinsic apoptotic pathway (Figure 3D and E). This pathway involves Bcl-2 family member proteins, which can be divided into anti-apoptotic, pro-apoptotic, and BH3-only proteins (30). To determine if the Bcl-2 family plays a role in gefitinib-induced apoptosis in Slug-knockdown PC9/gef cells, we examined the expression of Bcl-2, BcL-$x_{L,}$ Puma, Bad and Bim by Western blotting. Neither BH3-only proteins (Bad, Puma) nor anti-apoptosis proteins (Bcl-2, Bcl-xL) were significantly altered after gefitinib treatment of Slug-knockdown PC9/gef cells (Figure 5A). In contrast, the expression of three isoforms of Bim—Bim short (Bim_s), Bim long (Bim_L), and Bim extra long (Bim_{EL})—were greatly increased in si-Slug 3-transfected PC9/gef cells treated with gefitinib (Figure 5A). To explore the functional relationship between Slug and Bim, we transfected PC9/gef cells with both Slug siRNA and Bim siRNA, or with si-scramble, si-Slug 3, or Bim siRNA alone, and then treated cells with gefitinib. Co-knockdown of Slug and Bim significantly reduced the percentage of apoptotic cells after gefitinib treatment compared with knockdown of Slug alone (Figure 5B). Collectively, these results suggest that Slug suppresses the expression of pro-apoptotic Bim in gefitinib-treated cells. To confirm this, we treated Slug-overexpressing PC9-Slug cells with gefitinib and analyzed for the expression of Bim. As shown in Figure 5C and D, Bim expression following gefitinib treatment was decreased in PC9-Slug cells compared to PC9-mock cells; thus, gefitinib-induced Bim upregulation was

abolished by Slug. These results indicate that the depletion of Slug reversed the gefitinib resistance of PC9/gef through upregulation of Bim.

The expression of Slug increases after the development of acquired EGFR TKI resistance in lung cancer patients.

To confirm the role of Slug in EGFR TKI resistance, we collected cancer cells from 44 malignant pleural effusions of lung adenocarcinomas (24 sampled at the time of diagnosis of lung cancer and 20 sampled after the development of acquired resistance to EGFR TKI) for determination of Slug expression (31). The clinical characteristics of the 44 patients are listed in Supplementary Table E2. There were no differences in the clinical characteristics between the treatment-naïve patients and patients of tumors with acquired resistance to EGFR TKI.

Real-time quantitative RT-PCR was used to determine the amount of Slug mRNA. As shown in Figure 6A, Slug mRNA expression in lung adenocarcinomas after the development of acquired EGFR TKI resistance (1.43 ± 1.72, n = 20) was significantly higher than that before treatment (0.55 ± 0.56, n = 24; $p = 0.039$, Student's *t*-test).

In addition, we collected three paired patient samples (treatment-naïve and after development of acquired resistance to EGFR TKI) for analysis of Slug mRNA expression. These samples revealed a trend toward increased Slug expression after the development of acquired EGFR TKI resistance (Figure 6B). The EGFR mutation status and the duration of EGFR TKI use are listed in Supplementary Table E3.

DISCUSSION

There has been substantial recent development in molecular-targeted therapies, an available option in addition to conventional cancer treatments. Molecular-targeted therapy drugs can interfere with and block specific molecular pathways involved in cancer cell growth and progression (1, 3). Once such target that has been extensively studied is activated EGFR, which can be effectively blocked by EGFR TKIs. However, the development of resistance to EGFR TKIs continues to critically limit the long-term control of cancer using this strategy. In this study, we found that EMT correlates with resistance to gefitinib in lung cancer. Using gain- and loss-of-function approaches, we also showed that expression of the EMT regulator Slug is required for gefitinib resistance. Silencing Slug in cells with acquired gefitinib resistance restored gefitinib-induced apoptosis, a process that is mediated mainly through increased activation of Bim and caspase-9.

Resistance to EGFR TKIs in many cancers harboring wild-type EGFR is associated with loss of the epithelial marker E-cadherin (15-17), suggesting involvement of EMT in the resistance mechanism. Consistent with this, ectopic expression of E-cadherin in NSCLC cells, which possess a mesenchymal phenotype, enhances gefitinib sensitivity (18). Yauch et al. also reported that E-cadherin expression is a novel biomarker capable of predicting the clinical efficacy of erlotinib

in NSCLC patients (17). Cells of the A549 NSCLC cell line, which expresses wild-type EGFR and exhibits resistance to gefitinib, become more aggressive and more resistant to gefitinib upon loss of biomarkers associated with epithelial status and gain of biomarkers associated with mesenchymal status (32). These observations suggest that EMT contributes to primary resistance to gefitinib, although it should be noted that these studies do not completely mimic clinical reality because wild-type lung cancer cells are intrinsically resistant to gefitinib. Using two types of mutant EGFR-expressing cell lines—gefitinib-sensitive parental cells and derivative clones that had developed gefitinib resistance—we found that expression of E-cadherin was decreased and expression of vimentin and Slug were increased in cells with acquired resistance to gefitinib. Our results thus suggest that EMT contributes to acquired resistance to EGFR TKIs in EGFR-mutant, gefitinib-sensitive cells.

Expression of the EMT regulators Slug, snail, twist, and zeb-1, which cause EMT induction through transcriptional reprogramming, may differentially influence EGFR TKI resistance. Recently reports have shown that zeb-1 mRNA levels are inversely related to gefitinib sensitivity in NSCLCs that express wild-type EGFR (15, 18). Snail has also been shown to downregulate the transcription of E-cadherin and ErbB3, which contribute to gefitinib sensitivity (33-34). However, zeb-1 and snail expression were not significantly different between gefitinib-sensitive PC9 cells and

gefitinib-resistant PC9/gef cells, suggesting that zeb-1 and snail are not involved in the acquired resistance to gefitinib in lung cancer cells, although they may be involved in intrinsic resistance.

Slug, a member of the snail superfamily of zinc finger transcription factors, is the key EMT regulator responsible for conferring acquired resistance to EGFR TKIs in NSCLC cells. Our previous studies showed that the expression of Slug promotes the invasivity of tumor cells through increased activity of metalloproteinase-2 and suppression of E-cadherin (24). The mechanism of Slug-induced invasiveness depends on p53 status; notably, mutant p53 can stabilize Slug protein (35). A recent reported suggested that reducing the expression of Slug enhanced the sensitivity of neuroblastoma cell lines to imatinib mesylate, a soluble small-molecule tyrosine kinase inhibitor, by attenuating Bcl-2 expression (28). Slug can also antagonize p53-mediated apoptosis in hematopoietic progenitors by repressing Puma. Thus, Slug could regulate cancer cell survival via direct or indirect transcriptional regulation of proapoptotic and antiapoptotic genes (29, 36-37), although further study will be required to resolve the molecular details. Therefore, in addition to EGFR T790M and *MET* amplification, resistance to gefitinib may be mediated through Slug-induced EMT, which confers resistance by interfering with the balance between apoptosis and anti-apoptosis.

We screened for changes in Bcl-2 family members after knockdown of Slug and treatment with gefitinib. We found that only Bim was altered by depletion of Slug in the acquired gefitinib-resistance model after treatment with gefitinib, suggesting that Slug affected gefitinib sensitivity in cancer cells through changes in the expression of Bim. Specifically, treatment of Slug-knockdown PC9/gef cells with gefitinib resulted in increased expression of Bim, suggesting that Slug may suppress Bim expression in response to gefitinib. Consistent with this, we also found that both Bim protein and mRNA were decreased in Slug-overexpressing PC9-Slug cells following gefitinib treatment. Our results are in accord with previous reports, which have shown that Bim serves as an executioner of EGFR TKI-induced apoptosis in lung cancer cell lines containing activating EGFR mutants (38), and siRNA-mediated depletion of Bim causes insensitivity to gefitinib and erlotinib (39).

To the best of our knowledge, the role of Slug in cancer cells from patients treated with EGFR TKIs has not been explored. The present study showed that the mean mRNA levels of Slug expression were significantly higher in cancer cells from EGFR TKI-treated patients after the development of acquired resistance to EGFR TKIs than in treatment-naïve cancer cells. While the difference is not so dramatic, it is consistent with the results of our in vitro cell line model, in which Slug mRNA levels were increased in cells with acquired resistance to gefitinib (PC9/gef) compared with

parental cells (PC9). An increase in Slug expression was also noted in the three patients for whom paired samples were available for the analysis of Slug expression before and after EGFR TKI treatment. In addition to the presence of the T790M mutation, changes in Slug may prove to be useful for monitoring drug resistance in patients who have received EGFR TKI treatment, but due to the heterogeneity in the mechanisms of acquired resistance additional clinical studies are necessary to confirm this hypothesis.

An appropriate combination of gefitinib administration and Slug depletion might be a potential approach to lung cancer therapy. First, Slug suppression alone led to a degree of spontaneous apoptosis in PC9/gef cells. Second, Slug depletion appeared to markedly sensitize PC9/gef cells to gefitinib-induced apoptosis. Third, Slug supported EGFR-mutant tumor growth in an animal xenograft model, even with gefitinib treatment. Taken together, our data suggest that the development of Slug-targeted drugs is a promising strategy for enhancing the efficacy of molecular-targeted drugs and improving the effectiveness of lung cancer therapy.

Potential conflicts of interest

Dr. Jin-Yuan Shih received honoraria from AstraZeneca and Roche for talks. Dr. Chih-Hsin Yang received honoraria from AstraZeneca and Roche for talks and service on an ad hoc advisory committee. All other authors report no conflicts of interest.

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Figure Legends:

Figure 1. PC9/gef was resistant to gefitinib, but there was no T790M mutation or MET amplification

A, PC9 and PC9/gef cells were exposed to various doses of gefitinib for 5 days and assayed for survival by MTS assay. PC9 was sensitive to gefitinib treatment. PC9/gef was resistant to gefitinib. For each dose, six-repeat wells were treated. The curves are the mean survival data for three independent experiments. Error bars show the standard deviations. (***$P< 0.001$)

B, EGFR T790M (C to T alteration in 2369th nucleotide) detection by high sensitive MALDI-TOF nucleotide mass spectrometry. Shifted signals due to incorporated nucleotides (C or T) of un-extend detection probes (U) after single nucleotide extension reaction can be identified in the spectrum. There was no T790M mutation (T) detected in PC9, PC9/gef and its subclones (PC9/gef B4, C2, C4 and C7). H1975, lung adenocarcinoma cell line harboring mutant EGFR L858R and T790M, is a positive control. H1650, lung adenocarcinoma cell line harboring exon 19 deletion, is a negative control. The results shown are representative of three independent experiments.

C, The copy number of the *MET* gene was evaluated by real-time quantitative PCR of genomic DNA. No *MET* amplifications were noted in PC9/gef and its subclones. The

value represents the mean of triplicate for each subclone. The experiment was repeated three times with similar results.

Figure 2. The EMT regulator Slug played a role in gefitinib resistance.

A, The expression of E-cadherin, vimentin and α-tubulin was evaluated by Western blot (left). Immunofluorescence staining of vimentin, E-cadherin (green) and propidium iodide (blue) was analyzed by confocal microscopy (right). The results shown are representative of three independent experiments.

B, Matrigel invasion assay of PC9 and PC9/gef cells. Columns, number of cells invaded across the membrane. Data represent mean ± SD of three independent experiments. (**$P< 0.01$)

C, The expression of EMT-related regulators was evaluated by Western blot.

D, The expression of mRNA of Slug, snail, twist and zeb-1 was evaluated by quantitative real-time RT-PCR. Data represent mean ± SD of three independent experiments. (***$P< 0.001$)

Figure 3. Knockdown of Slug expression reversed gefitinib resistance in PC9/gef cells.

A, CL1-5 cells were transfected with different Slug siRNAs (si-Slug 1, si-Slug 2, si-Slug 3) or scramble siRNA (si-scramble). The expression of Slug was evaluated by

real-time RT-PCR. Data represent mean ± SD of three independent experiments. (**P< 0.01)

B, PC9/gef cells were transfected with si-Slug3 or si-scramble. The expression of E-cadherin mRNA was evaluated by real-time RT-PCR. Data represent mean ± SD of three independent experiments. (*P< 0.05)

C, The expressions of Slug, E-cadherin and vimentin was determined by Western blot analysis after transfection of PC9/gef cells with si-Slug3 or si-scramble. Lamin B and α-tubulin were as loading control. The results shown are representative of three independent experiments.

D, Cells were incubated with 0.05μM gefitinib for 40 h after transfection of si-scramble or si-Slug3. Cells were then stained with annexin V-FITC and propidium iodide, and analyzed by flow cytometry. The results shown are representative of three independent experiments.

E, The percentage of apoptotic cells was expressed as the sum of the bottom right (early state of apoptosis) and top right quadrants (late stage of apoptosis). The percentage of apoptotic cells from three independent experiments is shown. Error bars show the mean ± standard deviation. Data represent mean ± SD of three independent experiments. (***P< 0.001)

F and G, caspase 9 activity of these cells was analyzed by luminescent assay (F) and

Western blot (G). Data represent mean ± SD of three independent experiments. (***P< 0.001)

Figure 4. Over-expression of Slug protects PC9 cells from gefitinib-induced apoptosis

A, The expression of Slug was evaluated by Western blot in PC9-mock and PC9-Slug.

B, Cells was exposed to 0.5 μM gefitinib for 24 h and assayed for apoptosis using flow cytometry. The columns are the mean for three independent experiments. Error bars show the standard deviations. (***P< 0.001)

C and D, PC9-Slug and PC9-mock cells were incubated with 0.5 μM gefitinib for 16 h and then caspase 9 activity of these cells was measured by Western blot (C) and luminescent assay (D). The results shown are representative of three independent experiments. (**P< 0.01)

E, PC9-mock or PC9-Slug cells were injected into athymic nude mice subcutaneously. The mice were treated with gefitinib (10mg/kg/day) or vehicle for 20 days and their tumor growths were measured every five days (n = 9 mice per group). Bars represent standard error of the mean. Asterisks indicate statistically significant difference between PC9-mock with gefitinib treatment and PC9-mock with vehicle treatment groups. (**P< 0.01)

Figure 5. Role of Bim in gefitinib induced apoptosis of Slug-depleted PC9/gef cells.

A, Cells were incubated with 0.05μM gefitinib for 40 h after transfection of si-scramble or si-Slug3. Bim, bad, bcl-2, bcl-xl, puma expression of these cells were analyzed by Western blot. The results shown are representative of three independent experiments.

B, PC9/gef cells were transfected individually with si-scramble, si-Slug3, si-Bim or combination of si-Slug3 and si-Bim and analyzed by Western blot. Following transfection of siRNAs, PC9/gef cells were treated with 0.05μM gefitinib for 40 h and analyzed for apoptotic cells using flow cytometry. The data is presented as the average percentage of cells staining annexin V-FITC and propidium iodide from three independent experiments. (***$P< 0.001$)

C and D, Bim_{EL} expression of PC9, PC9-mock and PC9-Slug cells were analyzed by Western blot (C) and real-time RT-PCR (D) after treatment with gefitinib. The results shown are representative of three independent experiments. (**$P< 0.01$)

Figure 6. The expression of Slug increased after development of EGFR TKI resistance in lung cancer patients

A, Box plot of Slug mRNA expression in lung adenocarcinoma patients by real-time RT-PCR. Box shows median and interquartile range ± 95% confidence interval. Patient samples were tested in triplicate. (*$P < 0.05$)

B, The expression of Slug mRNA of lung cancer cells in three paired samples at initial diagnosis and after acquired EGFR TKI resistance.

Fig 1

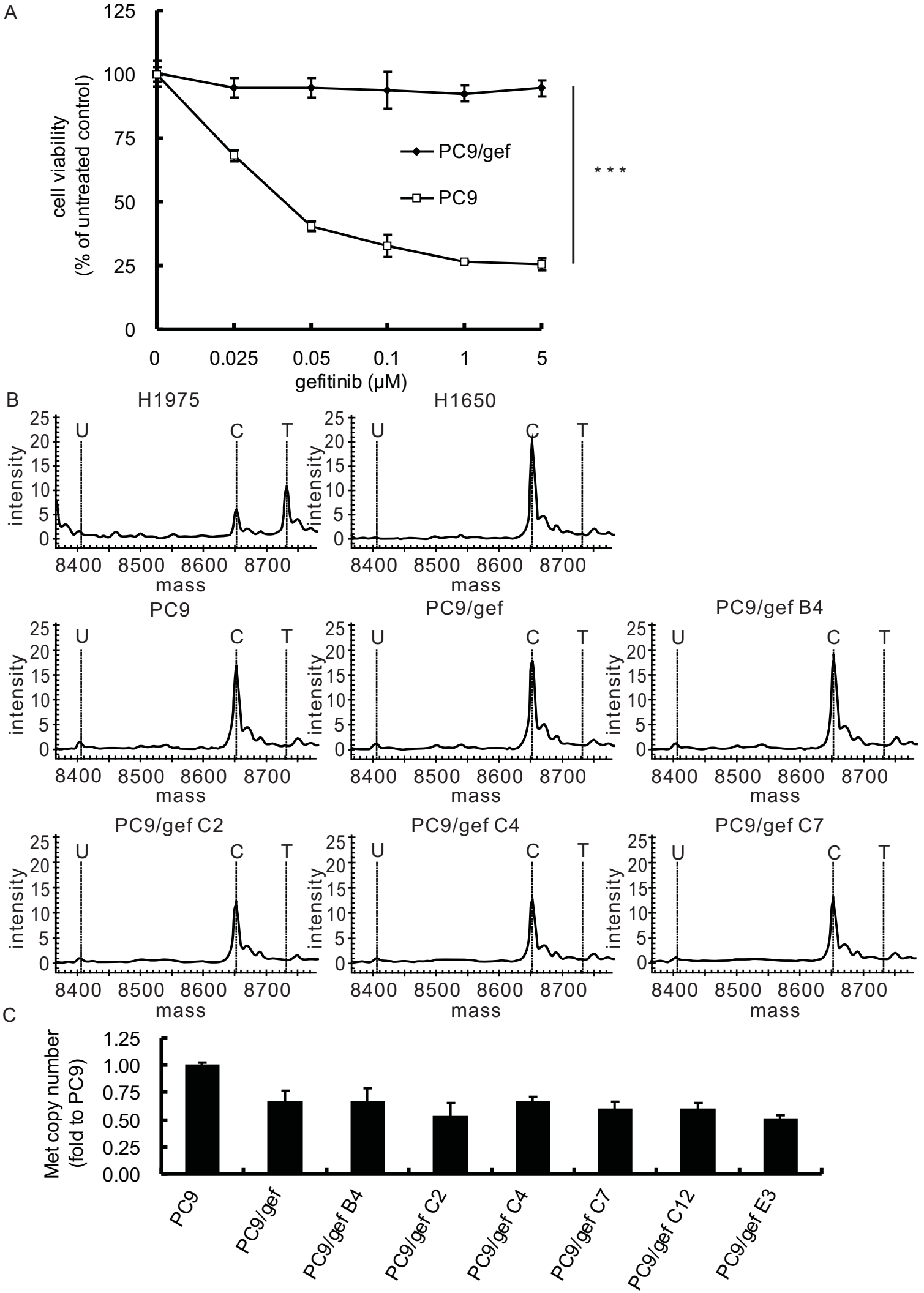
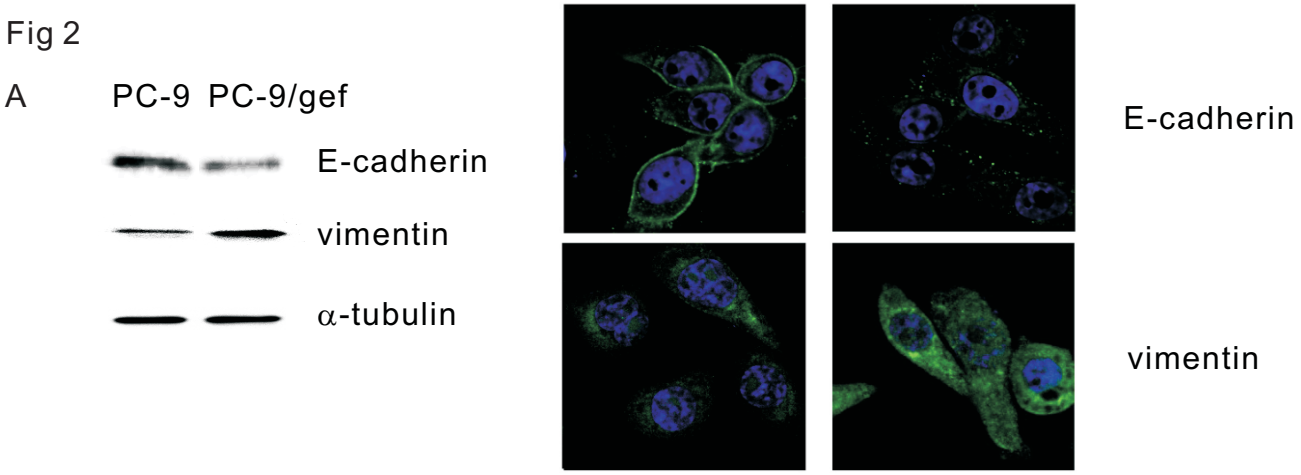
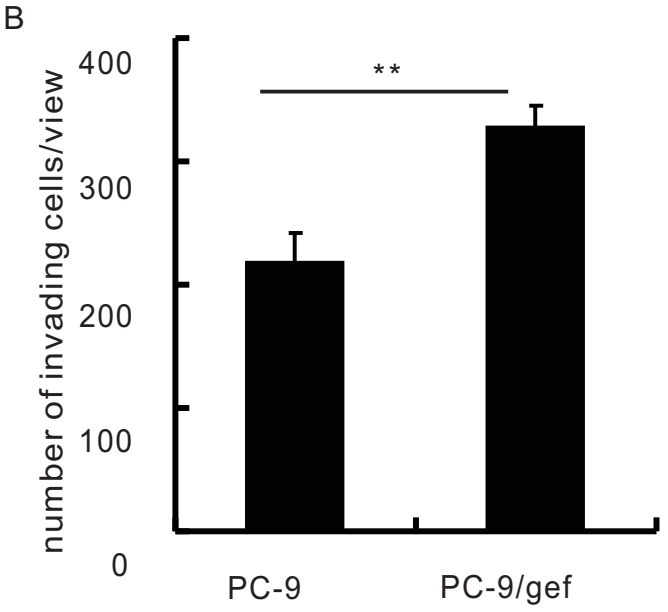
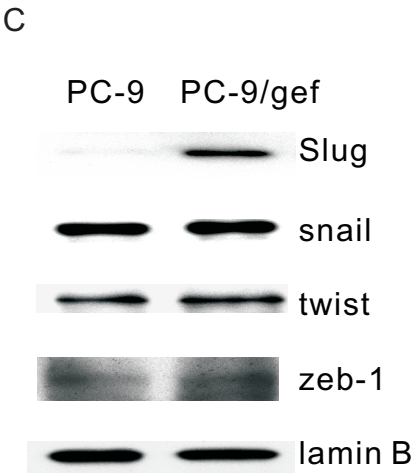

A
125
100
75
50
25
0
cell viability
(% of untreated control)
PC9/gef
PC9
* * *
0
0.025
0.05
0.1
1
5
gefitinib (μM)
B
H1975
H1650
U
C
T
intensity
mass
8400
8500
8600
8700
PC9
PC9/gef
PC9/gef B4
PC9/gef C2
PC9/gef C4
PC9/gef C7
C
Met copy number
(fold to PC9)
1.25
1.00
0.75
0.50
0.25
0.00
PC9
PC9/gef
PC9/gef B4
PC9/gef C2
PC9/gef C4
PC9/gef C7
PC9/gef C12
PC9/gef E3

Fig 2


A
PC-9
PC-9/gef
E-cadherin
vimentin
α-tubulin
E-cadherin
vimentin


B
number of invading cells/view
400
300
200
100
0
**
PC-9
PC-9/gef


C
PC-9
PC-9/gef
Slug
snail
twist
zeb-1
lamin B

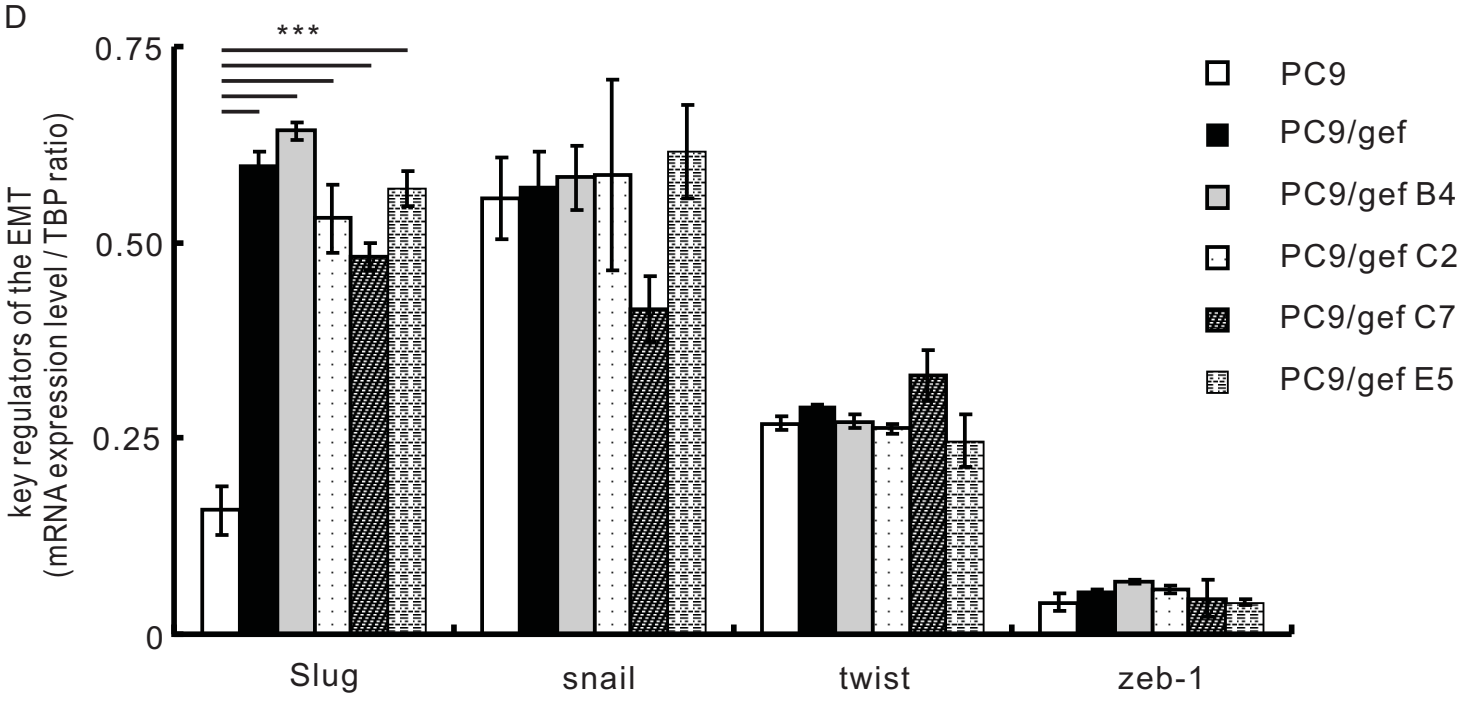

D
key regulators of the EMT
(mRNA expression level / TBP ratio)
0.75
0.50
0.25
0
***
Slug
snail
twist
zeb-1
PC9
PC9/gef
PC9/gef B4
PC9/gef C2
PC9/gef C7
PC9/gef E5

Fig 3

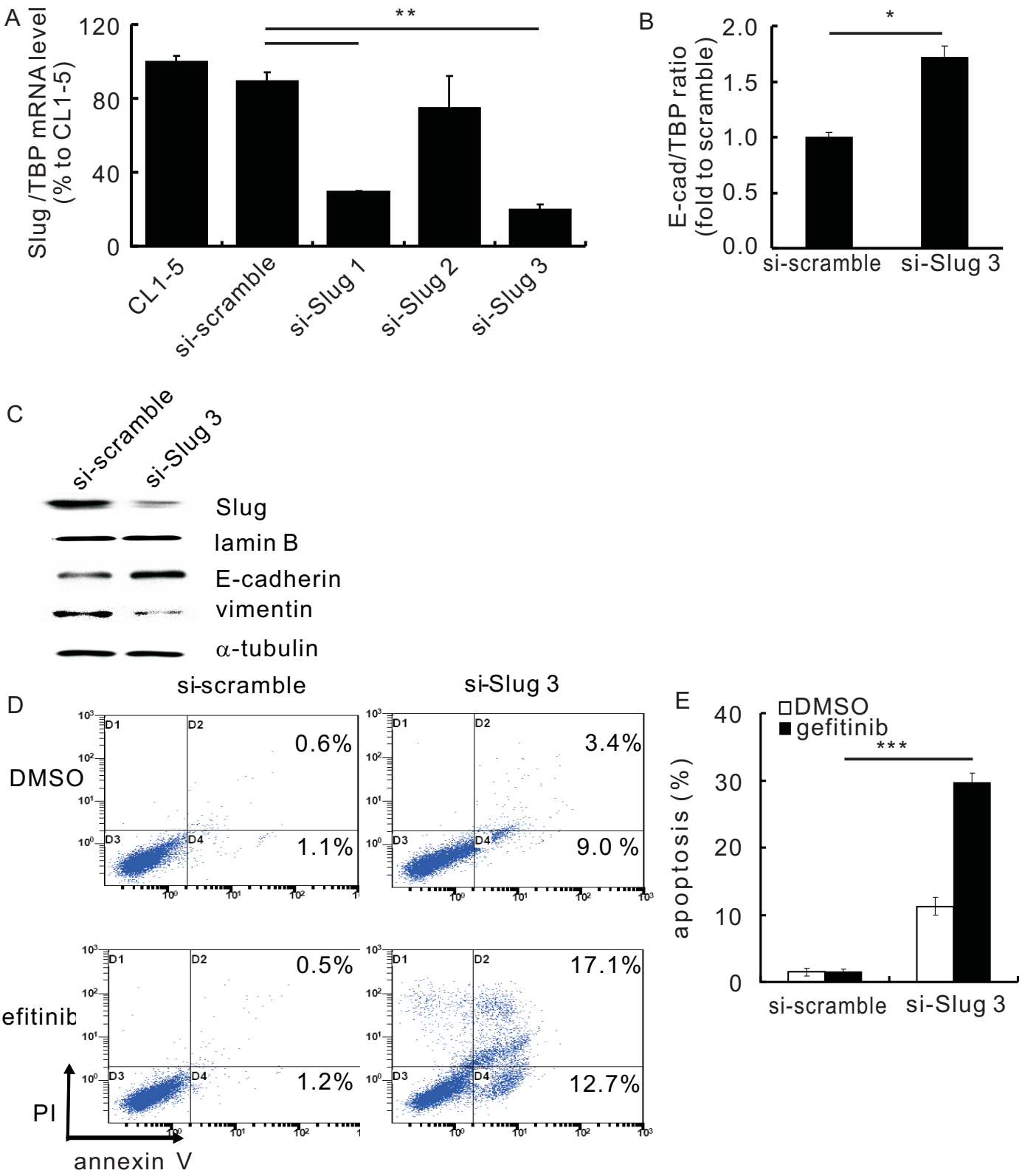

A
Slug /TBP mRNA level (% to CL1-5)
120
80
40
0
**
CL1-5
si-scramble
si-Slug 1
si-Slug 2
si-Slug 3
B
E-cad/TBP ratio (fold to scramble)
2.0
1.5
1.0
0.5
0.0
*
si-scramble
si-Slug 3
C
si-scramble
si-Slug 3
Slug
lamin B
E-cadherin
vimentin
α-tubulin
D
si-scramble
si-Slug 3
DMSO
gefitinib
0.6%
1.1%
3.4%
9.0 %
0.5%
1.2%
17.1%
12.7%
PI
annexin V
E
DMSO
gefitinib
apoptosis (%)
40
30
20
10
0
***
si-scramble
si-Slug 3
F
luminescence units (fold of PC9/gef)
4
3
2
1
0
DMSO
si-Slug 3
***
0
0.05
gefitinib (μM)
G
si-Slug 3
gefitinib
c-caspase 9
α-tubulin

Fig 4

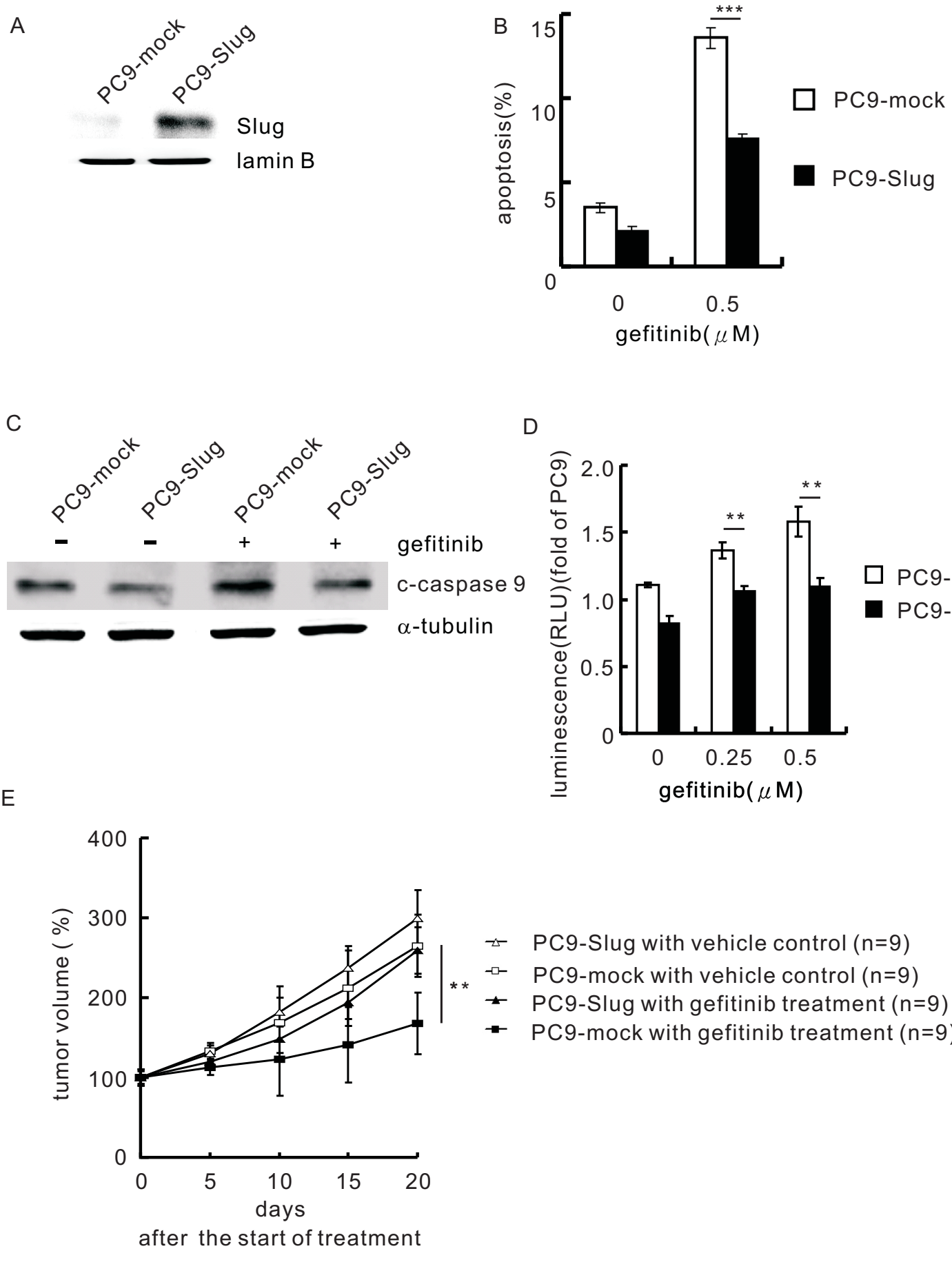

A
PC9-mock
PC9-Slug
Slug
lamin B
B
***
15
10
5
0
apoptosis(%)
PC9-mock
PC9-Slug
0
0.5
gefitinib(μM)
C
PC9-mock
PC9-Slug
PC9-mock
PC9-Slug
-
-
+
+
gefitinib
c-caspase 9
α-tubulin
D
2.0
1.5
1.0
0.5
0
luminescence(RLU)(fold of PC9)
**
**
PC9-mock
PC9-Slug
0
0.25
0.5
gefitinib(μM)
E
400
300
200
100
0
tumor volume (%)
**
0
5
10
15
20
days
after the start of treatment
PC9-Slug with vehicle control (n=9)
PC9-mock with vehicle control (n=9)
PC9-Slug with gefitinib treatment (n=9)
PC9-mock with gefitinib treatment (n=9)

Fig 5

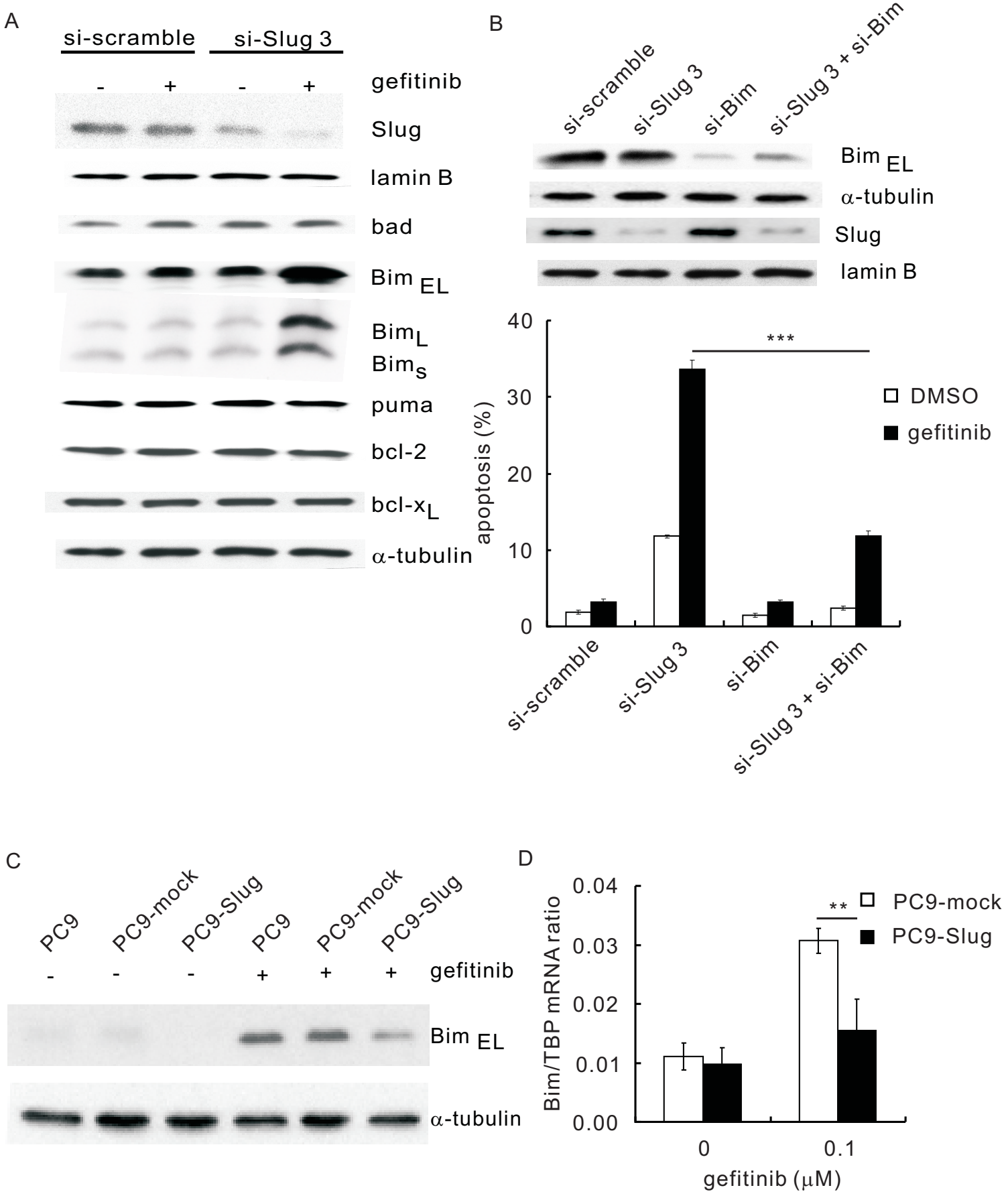
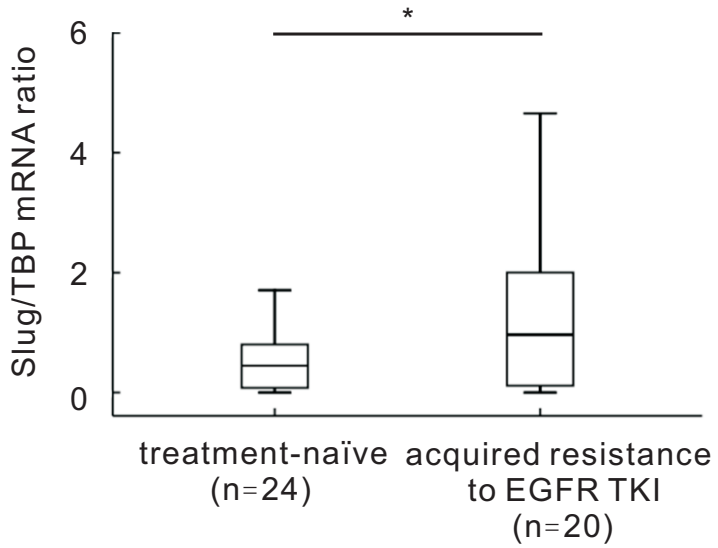
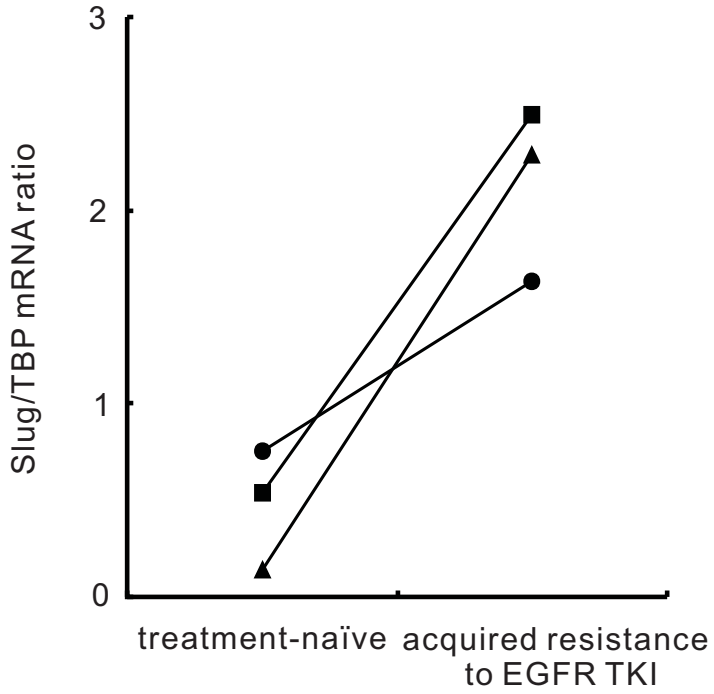

A
si-scramble
si-Slug 3
- + - +
gefitinib
Slug
lamin B
bad
Bim EL
Bim L
Bim S
puma
bcl-2
bcl-xL
α-tubulin
B
si-scramble
si-Slug 3
si-Bim
si-Slug 3 + si-Bim
Bim EL
α-tubulin
Slug
lamin B
apoptosis (%)
40
30
20
10
0
***
DMSO
gefitinib
si-scramble
si-Slug 3
si-Bim
si-Slug 3 + si-Bim
C
PC9
PC9-mock
PC9-Slug
PC9
PC9-mock
PC9-Slug
- - - + + + gefitinib
Bim EL
α-tubulin
D
Bim/TBP mRNA ratio
0.04
0.03
0.02
0.01
0.00
**
PC9-mock
PC9-Slug
0
0.1
gefitinib (μM)

Fig 6

A


*
6
4
2
0
Slug/TBP mRNA ratio
treatment-naïve
(n=24)
acquired resistance
to EGFR TKI
(n=20)

B


3
2
1
0
Slug/TBP mRNA ratio
treatment-naïve
acquired resistance
to EGFR TKI

Online Data Supplement

The Epithelial-Mesenchymal Transition Regulator Slug Confers Resistance to the Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor

Tzu-Hua Chang, Meng-Feng Tsai, Kang-Yi Su, Shang-Gin Wu, Cheng-Po Huang, Sung-Liang Yu, Yung-Luen Yu, Chou-Chin Lan, Chih-Hsin Yang, Shwu-Bin Lin, Chin-Pyng Wu, Jin-Yuan Shih, and Pan-Chyr Yang

Materials and Methods

EGFR Mutation Analysis Using a Sequenom MassARRAY® System

We first performed PCR on the cDNA region corresponding to the EGFR T790M mutation. After the PCR reaction, single-nucleotide extension was performed using a Typlex reagent kit and samples were cleaned up using SpectroClean Resin. Samples were then loaded onto the matrix of a SpectroCHIP® using a Nanodispenser (Matrix) and then analyzed by Bruker Autoflex MALDI-TOF MS. Data were collected and analyzed using Typer4 software (SEQUENOM, San Diego, CA, USA).

Antibodies, Western blotting, and Immunofluorescence Staining

The anti-Slug, anti-caspase 9, anti-bad, anti-puma, and anti-vimentin antibodies were purchased from Abcam (Cambridge, MA). The anti-bim antibody was purchased from Cell Signaling Technology (Danvers, MA). The anti-α-tubulin and anti-lamin B antibodies were purchased from Sigma-Aldrich (Saint Louis, MO). The anti-E-cadherin antibody was purchased from BD Biosciences (San Diego, CA). The preparation of cells lysates and analysis by Western blotting, and immunofluorescence staining were conducted as described previously (1).

Invasion Assay

The invasion assay was performed using 24-well Costar transwell chambers (Corning, NY) with 8-μm-pore-size membranes coated with a thin layer of BD

Matrigel (BD Biosciences). Cells were seeded onto the Matrigel-coated chamber, incubated for 20 h, and then removed from the upper surface of the filter by scraping with a cotton swab. The cells that adhered to the bottom of the membrane were stained with Giemsa solution.

Transfection of Short Interference RNA

The sequences of short interference RNA (siRNA) duplexes targeting human Slug were listed as below. Sequences of si-Slug 1 were 5'-GGACACAUUAGAACUCACAdTdT -3' (sense) and 5'-UGUGAGUUCUAAUGUGUCCdTdT -3' (antisense).; sequences of si-Slug 2 were 5'- CAAACAUAAGCAGCUGCACdTdT -3' (sense) and 5'- GUGCAGCUGCUUAUGUUUGdTdT -3' (antisense) ; sequences of si-Slug 3 were 5'-GGACCACAGUGGCUCAGAAdTdT-3' (sense) and 5'-UUCUGAGCCACUGUGGUCCdTdT-3' (antisense).

The Slug siRNA was synthesized from Dharmacon (Lafayette, CO). siGENOME SMARTpool reagent for human Slug were obtained from Dharmacon. Transfection was performed using LipofectAMINE 2000 reagent (Invitrogen Carlsbad, California), according to manufacturer's instructions. Conditions of transfections were optimized for the amounts of siRNA, and LipofectAMINE per well culture as follows: 50 nM of siRNA and 1.5 μL of LipofectAMINE 2000 Reagent. Growth medium were replaced

after 6 hours.

Cloning of stable transfectants

Slug constructions were generated by using the Slug cDNA as described in our previous report (2). Constitutively Slug overexpression clone were performed by introducing Slug plasmid into HCC827 cells by using LipofectAMINE reagent (Invitrogen). The selection of stable transfectant was used 200 μg/mL Gentamicin (G418; Invitrogen) in 3 to 4 weeks. HCC827-Slug9 and HCC827-Slug11 were selected as representatives of Slug overexpression clone. The mock vector-transfected cells (HCC827-mock) were used in bulk for the control.

Slug overexpression in PC9 cells was accomplished by infecting cells with lentiviruses containing the entire human Slug coding region, prepared using the ViraPower Lentiviral Expression System (Invitrogen, Carlsbad, CA), as described by the manufacturer.

Real-time Quantitative RT-PCR

Total mRNA was extracted using TRIzol reagent (Invitrogen). Relative Slug mRNA levels were determined by real-time RT-PCR using the TaqMan EZ RT-PCR Core Reagents and the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems), as previously described (2). Thermal cycling conditions for Slug were 2 min at 50°C, 30 min at 60°C and 5 min at 95°C, followed by 40 cycles of 15 s at

95°C and 1 min at 60°C.

cDNA was synthesized from total RNA by reverse transcription using Superscript II reverse transcriptase (Invitrogen), as described by the manufacturer. Briefly, a reaction mix containing 2 μg total RNA, 50 ng/μl random hexamers, 0.5 mM dNTP mix, 1x First-Strand Buffer, 10 mM DTT, 40 U/μl RNaseOUT, and 200 U/μl SuperScript II RT was incubated at 65°C for 5 min, chilled on ice for 5 min, incubated at 25°C for 10 min and then at 50°C for 50 min. Relative levels of mRNA of other target were investigated using a SYBR Green dye I-based real-time RT-PCR system. Reactions were performed on an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems, CA) using the following cycling conditions: 15 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. At the end of the PCR cycle, melting curve analyses were performed in order to validate specific generation of the expected PCR product. The Primer sequences are listed in Supplementary Table E1.

Real-time Quantitative PCR

DNA copy number was determined by amplifying 20 ng genomic DNA for *MET* and *MTHFR* (internal control) using the QuantiTect SYBR Green PCR kit (Qiagen) and the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems). Primer sequences for *MET* copy-number determinations are described in

a previous study (3).

Supplement References:

1. Wang SP, Wang WL, Chang YL, Wu CT, Chao YC, Kao SH, Yuan A, Lin CW, Yang SC, Chan WK, Li KC, Hong TM, Yang PC. P53 controls cancer cell invasion by inducing the mdm2-mediated degradation of slug. *Nat Cell Biol* 2009;11:694-704.

2. Shih JY, Tsai MF, Chang TH, Chang YL, Yuan A, Yu CJ, Lin SB, Liou GY, Lee ML, Chen JJ, Hong TM, Yang SC, Su JL, Lee YC, Yang PC. Transcription repressor slug promotes carcinoma invasion and predicts outcome of patients with lung adenocarcinoma. *Clin Cancer Res* 2005;11:8070-8078.

3. Bean J, Brennan C, Shih JY, Riely G, Viale A, Wang L, Chitale D, Motoi N, Szoke J, Broderick S, Balak M, Chang WC, Yu CJ, Gazdar A, Pass H, Rusch V, Gerald W, Huang SF, Yang PC, Miller V, Ladanyi M, Yang CH, Pao W. Met amplification occurs with or without t790m mutations in egfr mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc Natl Acad Sci U S A* 2007;104:20932-20937.

Supplementary Figure E1:

Sequencing chromatograms of EGFR T790M. PC9/gef and its subclones (PC9/gef B4 and PC9/gef C4) did not carry the T790M mutation. Arrow indicates the T790M mutation site (nucleotide C to T). H1975 is a positive control. H1650 is a negative control. The experiment was repeated three times with similar results.

Supplementary Figure E2:

A, PC9/gef cells were transfected with Slug siRNA 3 (si-Slug 3), pool-Slug siRNA (pool si-Slug) or scrambled siRNA (si-scramble). The expression levels of Slug and lamin B (loading control) were determined by Western blot analysis.

B, Cells were incubated with 0.05 μM gefitinib for 40 h after transfection of si-scramble or pool si-Slug. Cells were then stained with annexin V-FITC and propidium iodide, and analyzed by flow cytometry. The percentage of apoptotic cells from three independent experiments is shown in C. Error bars: standard deviation (***$P < 0.001$).

Supplementary Figure E3:

A, Overexpression of Slug in SK-MES-1 by transient transfection.

B, SK-MES-1 cells were transfected with increasing amounts of Slug expression vector, then exposed to 1 μM gefitinib for 5 days and assayed for cell death by MTS

assay (**P < 0.01). The results shown are representative of three independent experiments.

Supplementary Figure E4

A, The expression of Slug was evaluated by Western blot analysis in HCC827-mock cells and HCC827-Slug cells (two clones: HCC827-Slug9 and HCC827-Slug11). The results shown are representative of three independent experiments.

B, Quantitative real-time RT-PCR was used to evaluated E-cadherin mRNA levels in HCC827-mock and HCC827-Slug cells. Data represent mean ± SD of three independent experiments. (***P < 0.001)

C, Cells were exposed to vehicle, 0.1 μM gefitinib, or 1 μM gefitinib for 48 h and assayed for apoptosis using flow cytometry. Data represent mean ± SD of three independent experiments. (**P < 0.01)

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Supplementary Table E1: Primers used to amplify EMT regulators by real-time PCR

	Forward sequence (5'-3')	Reverse sequence (5'-3')
Snail	**CCCAGTGCCTCGACCACTAT**	**GCTGGAAGGTAAACTCTGGATTAGA**
Zeb-1	**TGACAGAAAGGAAGGGCAAGA**	**CAGGTGAGTAATTGTGAAAATGCAT**
Twist	**GCCGGAGACCTAGATGTCATTG**	**CACGCCCTGTTTCTTTGAATTT**
E-cad	**CCGAGAGAGTTTCCCTACGTATACC**	**CCCTTGTACGTGGTGGGATT**
TBP	**ACG CCA GCT TCG GAG AGT T**	**CCT CAT GAT TAC CGC AGC AAA**

Supplementary Table E2: The clinical characteristics of lung adenocarcinoma patients with malignant pleural effusions

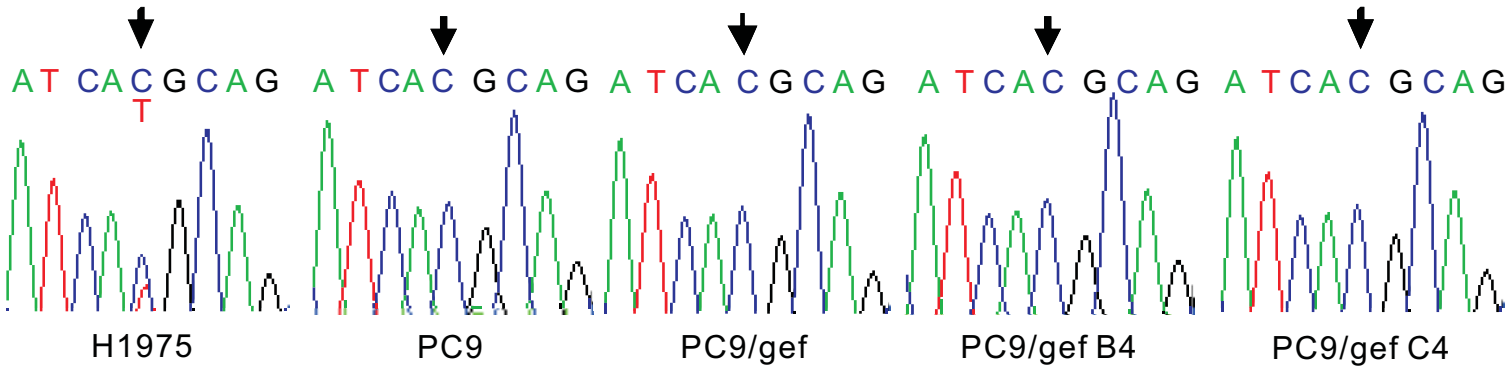
		Pleural effusion		
	Patient No.	treatment- naïve	Acquired resistance to EGFR TKI	*P*
Total No.	44	24	20	
Age, median years (range)	66.3 (30.1-89.2)	66.8 (43.7-89.2)	64.2 (30.1-84.9)	0.131#
Sex				0.149
Female	25	16	9	
Male	19	8	11	
Smoking				0.484
Non-smokers	33	19	14	
Smokers	11	5	6	
ECOG PS				0.198*
0-1	38	19	19	
2-4	6	5	1	
Stage				0.904
I	2	1	1	
III	3	2	1	
IV	39	21	18	

*****Derived using Fisher's exact test; # derived using Mann-Whitney U test

Supplementary Table E3: The clinical characteristics of three paired patients before treatment and after development of resistance to EGFR TKIs

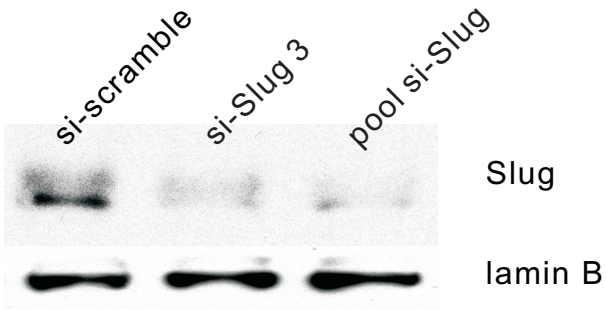
No.	Gender	Age	Mutation	EGFR TKI response	Duration of EGFR TKI use (mo.)
1	F	69.6	L858R	PR	6.2
2	M	61.4	L858R+E709G	PR	8.2
3	M	67	del-19	PR	14.9

Supplementary Fig E1



Supplementary Fig E2

A



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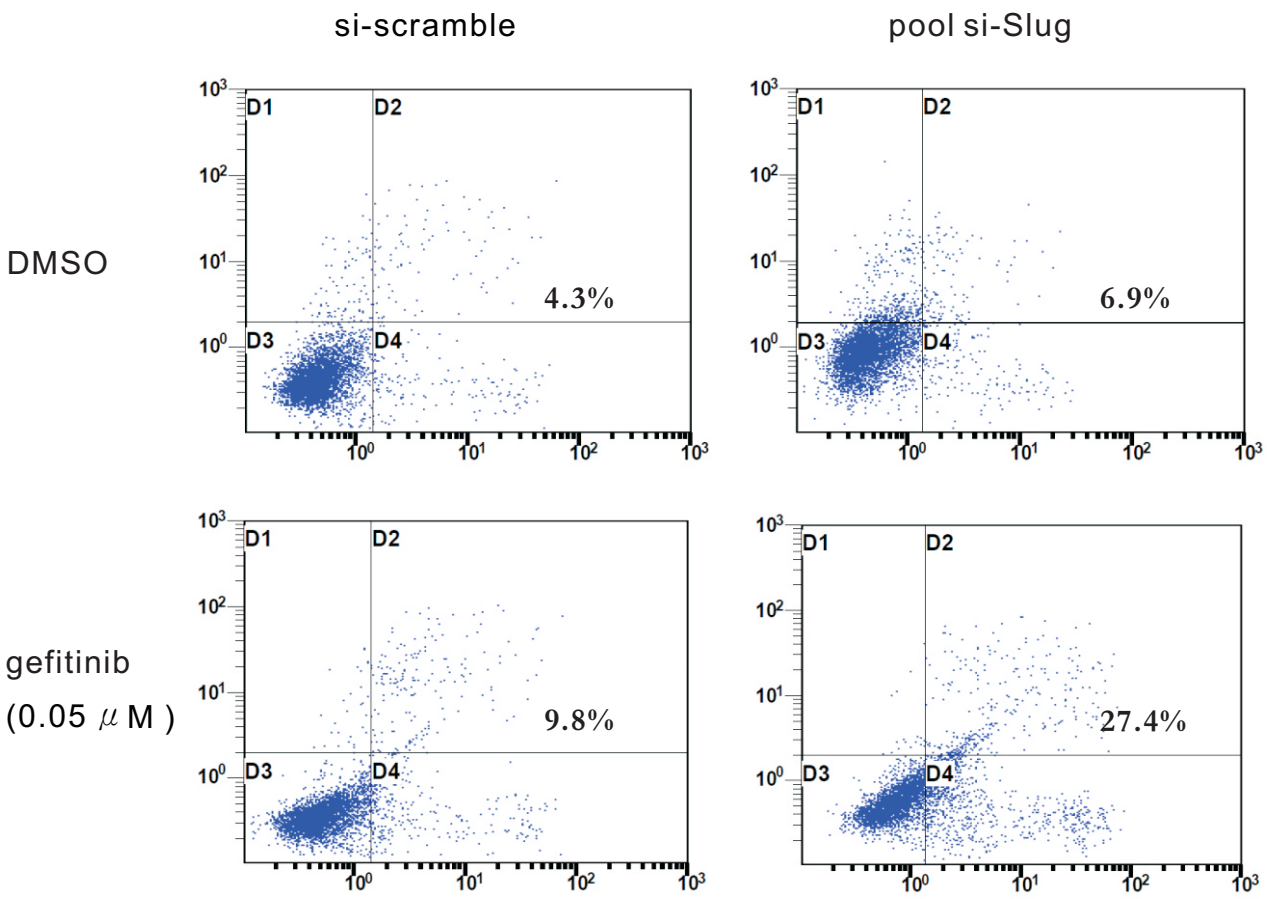

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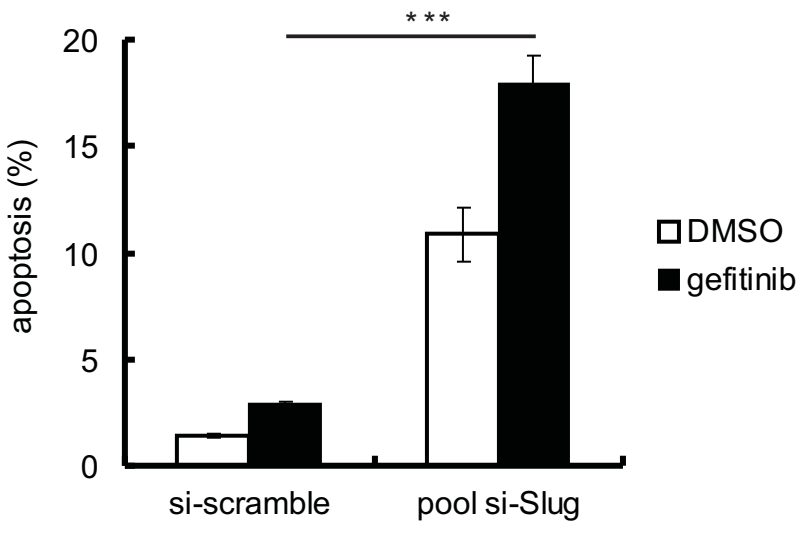

Supplementary Fig E3

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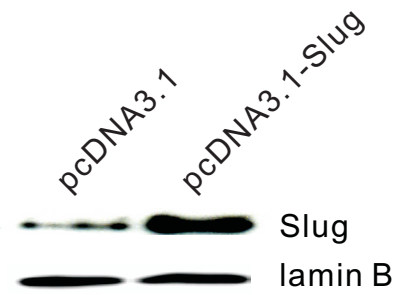

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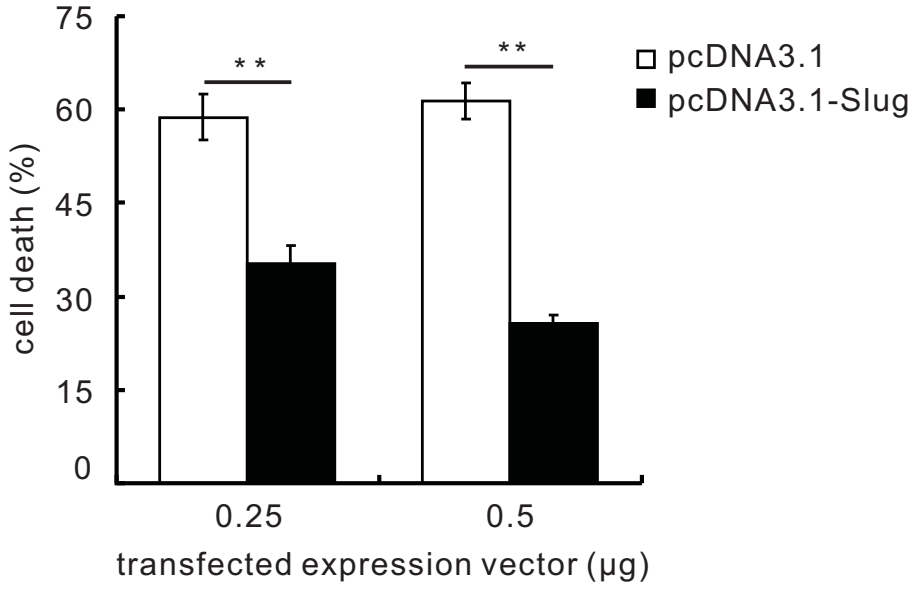

Supplementary Fig E4

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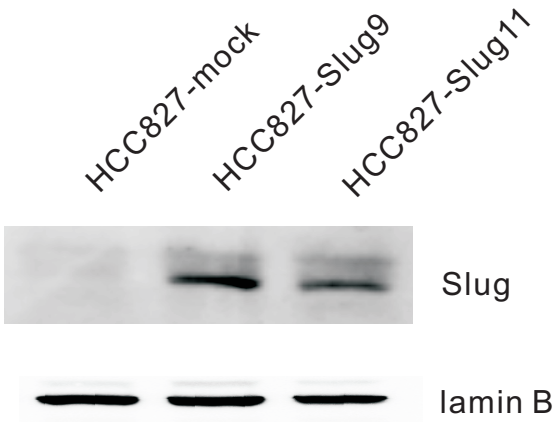

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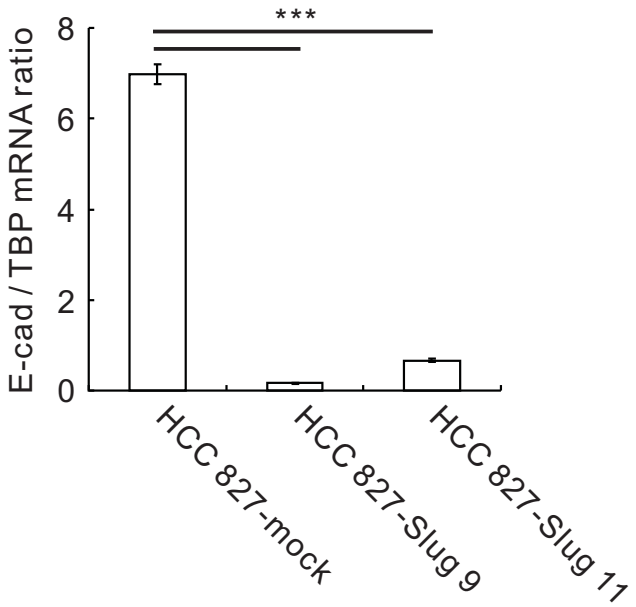

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