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MET copy number gain is associated with gefitinib resistance in leptomeningeal carcinomatosis of *EGFR*-mutant lung cancer

Shigeki Nanjo[1, 3]*, Sachiko Arai[1]*, Wei Wang[1,2], Shinji Takeuchi[1], Tadaaki Yamada[1], Akito Hata[3], Nobuyuki Katakami[3], Yasunori Okada[4,5], Seiji Yano[1].

[1]Division of Medical Oncology, Kanazawa University Cancer Research Institute, Kanazawa, Japan.

[2]Department of Radiation Oncology, Nanfang Hospital, Southern Medical University, Guangzhou, People's Republic of China.

[3]Division of Integrated Oncology, Institute of Biomedical Research and Innovation, Chuo-ku, Kobe, Japan.

[4]Department of Pathology, Keio University School of Medicine, Tokyo, Japan.

[5]Department of Pathophysiology for Locomotive and Neoplastic Diseases, Juntendo University Graduate School of Medicine, Tokyo, Japan.

* These authors contributed equally to this article.

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Corresponding Author: Seiji Yano, MD, PhD. Division of Medical Oncology Cancer Research Institute, Kanazawa University 13-1, Takaramachi, Kanazawa, Ishikawa, 920-0934, Japan

Phone: +81- 76-265-2794, Fax: +81- 76-244-2454 E-mail: syano@staff.kanazawa-u.ac.jp

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Abstract

Leptomeningeal carcinomatosis (LMC) occurs frequently in *EGFR*-mutant lung cancer, and develops acquired resistance to EGFR tyrosine kinase inhibitors (EGFR-TKIs). This study aimed to clarify the mechanism of EGFR-TKI resistance in LMC and seek for a novel therapeutic strategy. We examined *EGFR* mutations, including the T790M gatekeeper mutation, in 32 re-biopsy specimens from 12 LMC and 20 extracranial lesions of *EGFR*-mutant lung cancer patients who became refractory to EGFR-TKI treatment. All the 32 specimens had the same baseline *EGFR* mutations, but the T790M mutation was less frequent in LMC specimens than in extracranial specimens (8% vs. 55%, $p<0.01$). To study molecular mechanisms of acquired EGFR-TKI resistance in LMC, we utilized our previously developed mouse model of LMC with the *EGFR*-mutant lung cancer cell line PC-9/ffluc cells, in which acquired resistance to gefitinib was induced by continuous oral treatment. Compared with subcutaneously inoculated gefitinib-resistant tumors, the T790M mutation was less frequent in LMC that acquired resistance to gefitinib. PC-9/LMC-GR cells were established from the gefitinib-resistant LMC model, and they were found to be intermediately resistant to gefitinib and osimertinib (third generation EGFR-TKI). Although *EGFR*-T790M was negative, gefitinib resistance of PC-9/LMC-GR cells was related to *MET* copy number gain with MET activation. Moreover, combined use of EGFR-TKI and crizotinib, a MET inhibitor, dramatically regressed LMC with acquired resistance to gefitinib or osimertinib. These findings suggest that combination therapy with MET inhibitors may be promising for controlling LMC that acquires resistance to EGFR-TKIs.

Introduction

EGFR-activating mutations, including exon 19 deletions and L858R point mutations in exon 21, are present in a population of non-small cell lung cancer (NSCLC) (1, 2). *EGFR*-mutant lung cancer is frequently associated with central nervous system (CNS) metastasis, such as brain metastasis and leptomeningeal carcinomatosis (LMC), compared to NSCLC with wild type *EGFR* (3). EGFR tyrosine kinase inhibitors (EGFR-TKIs), first generation gefitinib/erlotinib and second generation afatinib, show remarkable clinical efficacy, with a response rate of 70–80% and median overall survival of 30 months (4-6). However, almost all patients acquire resistance to EGFR-TKIs, developing recurrent disease (7). EGFR-TKIs are generally effective for CNS metastases in *EGFR*-mutant lung cancer patients who are naïve to TKI treatment (8). While progression of CNS lesions is frequently observed during EGFR-TKI treatment (9), brain metastases are manageable by concomitant use of EGFR-TKI and radiation therapy including whole brain irradiation and stereotactic radiotherapy (10). There is, however, no established therapy for LMC, which is resistant to first and second generation EGFR-TKIs. Therefore, novel and effective therapies are needed for managing LMC in cancer patients.

For acquired resistance to EGFR-TKIs, several mechanisms, including secondary *EGFR* mutations, activation of alternative pathways, and histological transformation, have been identified. The most frequent secondary *EGFR* mutation is the T790M gatekeeper mutation, and T790M is detected in 50–60% of *EGFR*-mutant lung cancer cases with acquired EGFR-TKI resistance (11). Alternative pathways can be caused by MET activation by MET gene amplification (12) and its ligand HGF (13), AXL activation by Gas6, (14) and HER2 amplification (15). The transformation to small cell carcinoma (16) and the epithelial to mesenchymal transition (17) are also observed in an *EGFR*-mutant lung cancer population with acquired EGFR-TKI resistance. Involvement of these resistance mechanisms was shown in clinical specimens obtained mainly from extracranial tumor

lesions. Osimertinib is the third generation EGFR-TKI, which has activity toward EGFR with sensitive *EGFR* mutations, exon 19 deletions, L858R mutations, and T790M resistance mutations, but spares wild type EGFR (18). In a recent clinical trial, osimertinib demonstrated favorable antitumor activity and safety in *EGFR*-mutant lung cancer patients who were refractory to EGFR-TKIs (19). Thus osimertinib has been approved for EGFR-T790M-positive *EGFR*-mutant lung cancer patients in many countries, including US, EU, and Japan. On the other hand, mechanisms of acquired EGFR-TKI resistance in CNS are largely unknown. While poor penetration of EGFR-TKIs into the CNS due to the blood-brain barrier (BBB), termed pharmacodynamics resistance (20), and *EGFR*-D761Y intermediate-resistance mutation (21) can be involved in CNS resistance, the resistance mechanism to establish better therapeutics remain elusive.

We have previously established an *in vivo* imaging model of LMC by inoculating immunodeficient mice with *EGFR*-mutant lung cancer cells (22). Using this model, we found that erlotinib delayed LMC progression, but mice with LMC acquired resistance to erlotinib (22). While second generation afatinib and third generation osimertinib had higher activity than erlotinib in delaying LMC progression, the mice with LMC also acquired resistance to these next-generation EGFR-TKIs (22). These results resemble the clinical course of *EGFR*-mutant lung cancer patients with LMC treated with EGFR-TKIs, indicating the clinical relevance of our LMC model as a preclinical model for evaluating targeted drugs for LMC.

In the present study, we examined the incidence of *EGFR*-T790M and D761Y mutations in clinical LMC specimens and compared these tissues with clinical specimens from extracranial tumor lesions of EGFR-TKI-refractory *EGFR*-mutant lung cancer. We further explored mechanisms of acquired resistance to gefitinib by utilizing resistant tumor cells established from a gefitinib-resistant LMC model.

Materials and Methods

Patients and *EGFR* mutation analysis of re-biopsy specimens

Between December 2005 and April 2011, *EGFR* mutational analysis was performed in 622 tumor samples from 599 patients with NSCLC at the Institute of Biomedical Research and Innovation (Kobe, Japan). Of these patients, mutation status after EGFR-TKI failure was reconfirmed by re-biopsy in 28 patients (32 samples). Twelve of the samples were carcinoma cells obtained by lumbar puncture. Twenty extra-CNS tumor tissue or cell samples were obtained from lung metastases or malignant pleural effusions by several methods: ultrasound- or computed tomography-guided percutaneous needle biopsy, transbronchial biopsy with bronchoscopy, and thoracentesis. All patients were identified from medical records at the Institute of Biomedical Research and Innovation.

Informed consent for *EGFR* mutation analysis of re-biopsy specimens was obtained from all patients. We isolated tumor DNA from various specimens, and *EGFR* mutations within exons 18 to 21 were examined using the peptide nucleic acid (PNA)-locked nucleic acid (LNA) polymerase chain reaction (PCR) clamp method established by Nagai et al. (23). Exon 19 D761Y was tested by DNA sequencing as reported by Balak et al. (21).

Cell cultures and reagents

The *EGFR*-mutant human lung adenocarcinoma cell line PC-9 with a deletion in *EGFR* exon 19 (del E746_A750) and the human lung squamous cell carcinoma cell line EBC-1 with a *MET* amplification were purchased from Immuno-Biological Laboratories Co. (Gunma, Japan) and JCRB Cell Bank (Osaka, Japan), respectively. The human lung embryonic fibroblast line MRC-5 was obtained from RIKEN Cell Bank (Ibaraki, Japan). A PC-9 ffLuc-cp156 transfectant line (PC-9/ffluc) was established as previously described (24). These cells were maintained in RPMI-1640 medium

supplemented with 10% fetal bovine serum (FBS) and antibiotics. All cells were passaged for less than 3 months before renewing frozen, early-passage stocks. Cells were regularly screened for mycoplasma contamination using MycoAlert Mycoplasma Detection Kits (Lonza, Rockland, ME). The cell lines were authenticated at the laboratory of the National Institute of Biomedical Innovation (Osaka, Japan) by short tandem repeat analysis in May 2015. Gefitinib, osimertinib, crizotinib, and golvatinib were obtained from Selleck Chemicals (Houston, TX).

Tumor cell inoculation in severe combined immunodeficiency (SHO-Prkdc^{scid}Hr^{hr}) mice

We used 6-week-old female SHO-Prkdc^{scid}Hr^{hr} mice (SHO-SCID mice from Charles River, Yokohama, Japan) for the study. For the LMC model, mouse scalps were sterilized with 70% ethanol and PC-9/ffluc (1×10^4) cells were injected into the leptomeningeal space (between the external occipital protuberance and first cervical vertebra) with a 27G needle (22). For the subcutaneous tumor model, PC-9/ffluc (3×10^6) cells were implanted subcutaneously into the flanks of each mouse.

In the LMC model, tumor quantity was tracked in live mice by repeated noninvasive optical imaging of tumor-specific luciferase activity using the IVIS Lumina XR Imaging System (PerkinElmer, Alameda, CA), as described previously (22). The intensity of the bioluminescence signal was analyzed using Living Image 4.0 software (PerkinElmer) by serially quantifying the peak photon flux in the selected region of interest (ROI) within the tumor. The intensity of the bioluminescence signal was corrected for the total area of the ROI and elapsed time during which bioluminescence signals were read by the CCD camera, and this value was expressed as photons/s/cm^2/sr. The size of subcutaneous tumors and body weights of the mice were measured twice per week, and tumor volume was calculated in mm^3 (width^2 × length/2).

This study was carried out in strict accordance with the recommendations of the Guide for the

Care and Use of Laboratory Animals by the Ministry of Education, Culture, Sports, Science, and Technology in Japan. The protocol was approved by the Committee on the Ethics of Experimental Animals and the Advanced Science Research Center, Kanazawa University, Kanazawa, Japan (approval no. AP-153499). All surgeries were performed on mice anesthetized with sodium pentobarbital, and efforts were made to minimize animal suffering.

Cell viability assay

Cell viability was measured by an MTT assay. Tumor cells (2×10^3 in 100 μL RPMI 1640 plus 10% FBS) were plated per well in 96-well plates and incubated for 24 hours. EGFR-TKIs and/or MET-TKIs were then added to each well, and incubation was continued for another 72 hours. Cell growth was measured with MTT solution (2 mg/mL; Sigma, St. Louis, MO), as described (25).

Immunoblot analyses

Lysates were prepared using Cell Lysis Buffer (Cell Signaling, Danvers, MA). Immunoblotting was performed as previously described (13). Antibodies used in this study are summarized in Supplementary Table S1. Human phospho-RTK arrays were obtained from R&D Systems and used according the manufacturer's instructions.

Fluorescence *in situ* hybridization (FISH)

The MET 7q31.2 chromosomal locus was labeled with LSI MET Spectrum Red Probe (Abbott, Abbott Park IL, USA). Centromere 7 labeled with Spectrum Green Probe (CEP7(D7Z1), Abbott) was paired to control for copy number. FISH was performed using standard methods (26). Only nuclei with unambiguous CEP7 signals were scored for MET signal number.

Gene copy number analysis

Genomic DNA was extracted by a DNeasy Blood & Tissue Kit (QIAGEN, Tokyo). *MET* copy number was analyzed using a TaqMan gene copy number assay (ViiA realtime PCR system, Assay ID: Hs01432482_cn, Applied Biosystems, Yokohama, Japan), according to the manufacturer's instructions. TaqMan Copy Number Reference Assay, human RNase P was used as the endogenous reference gene. Fold increase in copy number was calculated as the ratio of the *MET* signal in each cell line to the ratio obtained in MRC-5 cells.

siRNA knockdown

Cells were seeded into 6-well plates at a density of 1-2 × 10^5 cells/well. Twenty-four hours later, cells were transfected with 5 nM of two siRNAs against *MET* (Dharmacon, Lafayette, CO) or Stealth RNAi-negative control low GC Duplex #3 (Invitrogen,Yokohama, Japan) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Transfected cells were cultured at 37°C for 5 days before analysis.

Statistical analysis

All analyses were performed using StatView software, Ver. 5.0 (SAS Institute Inc., Cary, NC, USA). A Fisher's exact test was used to analyze the difference between LMC- and extracranial-acquired resistance mutation status. A *P* value less than 0.05 was considered statistically significant.

Results

***EGFR*-T790M mutation was less frequent in LMC compared with extra-CNS tumors that acquired resistance to EGFR-TKIs, in *EGFR*-mutant lung cancer patients.** We obtained re-biopsy specimens from 28 patients with *EGFR*-mutant lung cancer who acquired resistance to gefitinib or erlotinib. Patient characteristics are provided in Supplementary Table 1. Among the 28 patients, 4 provided both LMC and extra-CNS samples after EGFR-TKI failure. All 12 LMC samples were obtained from cerebrospinal fluid. For the extra-CNS samples, 9 and 11 samples were obtained from lung tissues and malignant pleural effusions, respectively.

Baseline *EGFR*-sensitive mutations obtained by re-biopsy were all the same before EGFR-TKI treatment in both groups. Exon 19 deletion, exon 21 L858R mutation, and exon 19 G719A mutation in *EGFR* were detected in 4 (33%), 7 (58%), and 1 (8%) LMC samples, and in 6 (30%), 12 (60%), and 2 (10%) extra-CNS samples, respectively (Fig. 1). Exon 20 T790M resistance mutation was less frequent in the LMC group (1 of 12 samples, 8%) than in the extra-CNS group (11 of 20 samples, 55%) ($P = 0.01$). None of the 12 LMC samples had exon 19 D761Y, a moderate-resistance mutation discovered in a clinical brain metastasis specimen with acquired EGFR-TKI resistance (21) (Fig. 1). These results indicate that *EGFR*-T790M resistance mutations are less frequent in LMC, compared with extra-CNS tumors that acquired resistance to EGFR-TKIs in *EGFR*-mutant lung cancer patients.

***EGFR*-T790M resistance mutation is less frequent in an LMC model with *EGFR*-mutant lung cancer cells.** We previously established an *in vivo* LMC imaging model of *EGFR*-mutant lung cancer cells that express luciferase (PC-9/ffluc) (22). Utilizing this model, we obtained four LMC specimens with PC-9/ffluc cells that acquired gefitinib resistance after continuous oral treatment with gefitinib (Fig. 2A). We also obtained four subcutaneous tumor specimens, as extra-CNS tumors,

that acquired gefitinib resistance after the same treatment (Fig. 2B). While *EGFR* exon 19 deletions were observed in all four samples from the LMC model, neither *EGFR*-T790M nor D761Y mutations was detected in these samples. On the other hand, *EGFR* exon 19 deletion and T790M mutations, but not D761Y mutations, were detectable in all four subcutaneous tumor samples (Fig. 2C). These results of decreased frequency of T790M mutation in LMC resembled the findings in the clinical specimens, indicating the clinical relevance of our LMC model for analyzing molecular mechanisms of EGFR-TKI resistance.

To establish tumor cell lines from gefitinib-resistant LMC tumors, we made four cultures from four different mice with gefitinib-resistant LMC. We successfully obtained only one culture that grew in *in vitro* conditions; we referred to these as PC-9/LMC-GR cells. We examined the molecular mechanism of gefitinib resistance in PC-9/LMC-GR cells.

PC-9/LMC-GR cells have *MET* copy number gain and show intermediate resistance to gefitinib. *In vitro,* PC-9/LMC-GR cells were intermediately resistant to gefitinib compared with PC-9/ffluc cells (Fig. 3A). *EGFR*-T790M mutations were not detected in PC-9/LMC-GR cells. Phosphotyrosine array analyses revealed that MET phosphorylation was increased in PC-9/LMC-GR cells compared with PC-9/ffluc cells (Fig. 3B). We confirmed increased MET phosphorylation in PC-9/LMC-GR cells, compared with PC-9/ffluc cells, by immunoblot (Fig. 3C). MET protein levels were increased in PC-9/LMC-GR cells compared with those in PC-9/ffluc cells (Fig. 3C). In PC-9/ffluc cells, phosphorylation of EGFR and its downstream molecules AKT and ERK was inhibited by gefitinib. In PC-9/LMC-GR cells, while EGFR phosphorylation was inhibited, increased MET phosphorylation or AKT phosphorylation was not discernibly inhibited by gefitinib. These results suggest that MET activation may be involved in gefitinib resistance in PC-9/LMC-GR cells.

MET can be activated by various factors, including *MET* mutations, *MET* amplification, and

activation by its ligand HGF. We therefore examined how MET is activated in PC-9/LMC-GR cells. PC-9/LMC-GR cells did not produce detectable HGF levels (Supplementary Fig. 1A). We did not detect MET exon 14 skipping mutations (Supplementary Fig. 1B) or known kinase domain mutations, (Supplementary Fig. 1C). Primers used for amplifying MET gene were shown in Supplementary Table 2. Interestingly, FISH and PCR analyses of *MET* showed that *MET* copy number was increased in PC-9/LMC-GR cells compared with PC-9 or PC-9/ffluc cells (Fig 3 D, E). PC-9/LMC-GR cells were also intermediately resistant to osimertinib, a third generation EGFR-TKI with activity toward EGFR with the T790M mutation (Fig 3F).

We next evaluated the effect of MET inhibitors to assess whether MET activation is responsible for the intermediate resistance to gefitinib seen in PC-9/LMC-GR cells. Crizotinib and golvatinib (E7050), both of which have inhibitory activity toward MET (27, 28), sensitized PC-9/LMC-GR cells to gefitinib (Fig 4A). Immunoblotting of PC-9/LMC-GR cell lysates showed that while phosphorylation of EGFR and ERK was inhibited remarkably, AKT phosphorylation was suppressed slightly by gefitinib alone. Crizotinib or golvatinib alone inhibited MET phosphorylation remarkably and AKT phosphorylation discernibly. Gefitinib plus crizotinib or golvatinib inhibited the phosphorylation of EGFR and MET almost completely, and inhibited phosphorylation of AKT and ERK markedly (Fig. 4B). We further explored the effect of MET knockdown using siRNA specific for MET. MET siRNA (si-MET) successfully knocked down MET protein expression, but not EGFR expression (Fig. 4C). Under these experimental conditions, gefitinib plus treatment with MET siRNA inhibited phosphorylation of EGFR and MET almost completely, and inhibited phosphorylation of AKT and ERK remarkably (Fig. 4C). Moreover, treatment with MET siRNA sensitized PC-9/LMC-GR cells to gefitinib (Fig. 4D). Collectively, these results indicate that PC-9/LMC-GR cells acquired gefitinib resistance via MET activation associated with an increase in *MET* copy number.

A similar phenomenon was observed in other *EGFR*-mutant lung cancer cells, i.e., HCC827 with an *EGFR* exon 19 deletion. We inoculated luciferase-transfected HCC827 cells (HCC827/luc) in the leptomeningeal space of SHO-SCID mice. After the development of LMC, we administered daily gefitinib treatment and induced gefitinib resistance in the LMC model. Tumor cells recovered from the LMC sample were designated HCC827/LMC-GR. These cells were moderately more resistant to gefitinib compared with HCC827/luc cells. The *MET* copy number was two times higher in HCC827/LMC-GR cells than HCC827-luc cells. A MET inhibitor, golvatinib, partially sensitized the HCC827/LMC-GR cells to gefitinib. These results suggest that the *MET* copy number increase was associated, at least in part, with intermediate gefitinib resistance in HCC827/LMC-GR cells (Supplementary Figure 2).

Crizotinib overcomes acquired resistance to gefitinib and osimertinib in LMC models. We finally examined whether combination treatment with a MET inhibitor overcomes acquired gefitinib resistance in an LMC model (Fig. 5). To reproduce the acquired resistance seen in patients, we inoculated PC-9/ffluc cells into the leptomeningeal space of SHO-SCID mice, and treated the mice continuously with gefitinib to induce acquired resistance. Upon acquisition of gefitinib resistance, we randomized the mice and treated them further with gefitinib alone, crizotinib alone, or gefitinib plus crizotinib. Neither gefitinib nor crizotinib alone suppressed LMC progression. However, combination treatment with gefitinib plus crizotinib regressed LMC and successfully controlled the disease for over 50 days. These results suggest that combined treatment with crizotinib may be useful for treating LMC with acquired gefitinib resistance caused by *EGFR*-mutant lung cancer.

We further evaluated the efficacy of crizotinib on osimertinib resistance in our LMC model. PC-9/LMC-GR cells were injected into the leptomeningeal space of SCID mice (Fig. 6). The mice were treated daily with gefitinib or osimertinib. Despite gefitinib treatment, the mice inoculated with

PC-9/LMC-GR cells experienced LMC progression much more rapidly, compared with the mice inoculated with PC-9/ffluc cells, confirming low the sensitivity to gefitinib of PC-9/LMC-GR cells in LMC. While treatment with osimertinib delayed LMC progression, the mice inoculated with PC-9/LMC-GR cells experienced LMC progression by day 46, indicating acquired resistance to osimertinib. Combination treatment with crizotinib starting on day 48 remarkably regressed LMC which was refractory to osimertinib monotherapy. These results indicate that crizotinib may also be useful for controlling LMC with acquired resistance to osimertinib.

Discussion

The *EGFR*-T790M gatekeeper mutation is the most common cause of acquired resistance to EGFR-TKIs in *EGFR*-mutant lung cancer. In the present study, however, we found that the *EGFR*-T790M mutation is less frequent in LMC samples from *EGFR*-mutant lung cancer patients who were refractory to the EGFR-TKIs gefitinib or erlotinib. This phenomenon was confirmed in an acquired gefitinib-resistance LMC model using *EGFR*-mutant lung cancer cell lines. We further demonstrated that *MET* copy number gain associated with MET activation is involved in acquired gefitinib resistance in our LMC model. Moreover, we showed that the combined treatment with crizotinib, which inhibits MET, dramatically regresses LMC with acquired gefitinib resistance.

In PC-9/LMC-GR cells established from the LMC model with acquired resistance to gefitinib, MET activation was involved in intermediate gefitinib resistance. The *MET* copy number in PC-9/LMC-GR cells was 8, which represents a 2-fold increase compared to PC-9 and PC-9/ffluc cells. Engelman et al. established gefitinib-resistant *EGFR*-mutant lung cancer cell lines HCC827 GR1 and HCC827 GR2, with *MET* amplification by long-term gradual escalation of gefitinib *in vitro* (12). The *MET* copy number in these resistant cells was about 10-12 (12). Suda et al. also established

the erlotinib-resistant HCC827 cell line, HCC827ER, by this gradual escalation method, and showed that HCC827ER cells were re-sensitized to EGFR-TKIs by MET inhibitors (29). Their data showed that the *MET* copy number in HCC827ER cells was approximately 6-8 (29). Therefore, our results in PC-9/LMC-GR cells were not inconsistent with the results of these recent studies. According to the literature, the penetration rate of first generation EGFR-TKIs, such as gefitinib and erlotinib, to the CNS is low (1–3%) (30, 31). MET activation caused by this degree of *MET* copy number gain may be sufficient to confer resistance to low concentrations of EGFR-TKIs in LMC. In fact, PC-9/LMC-GR and PC-9/ffluc cells had similar sensitivity to gefitinib in the SC model (Supplementary Figure 3). Collectively, differences in the EGFR-TKI concentration between the tumors may explain differences in the mechanism by which resistance is acquired in LMC and extra-CNS tumors. Mechanisms conferring high resistance, such as the *EGFR*-T790M mutation, may be necessary for resistance in extra-CNS space where EGFR-TKIs penetrate effectively. However, intermediate resistant mechanisms, including *MET* copy number gains, may be sufficient for resistance in the CNS, where EGFR-TKIs exhibit poor penetration.

We demonstrated that combination treatment with crizotinib overcame acquired resistance to EGFR-TKIs in an LMC model with *EGFR*-mutant lung cancer. In our LMC model inoculated with PC-9/ffluc (EGFR-TKI sensitive) cells (Fig. 5A), acquired gefitinib resistance was induced by continuous treatment, and then tested with crizotinib. Since this model resembles the clinical course of *EGFR*-mutant lung cancer with LMC, it is considered to be a valuable preclinical model. Crizotinib is a kinase inhibitor approved for lung cancer with ALK fusion or ROS-1 gene fusions (32, 33), which also inhibits MET (34). Crizotinib, given as a first-line TKI treatment, has a promising effect on CNS metastasis in ALK fusion-positive lung cancer (35, 36). The CNS is, however, a frequent site of disease progression in patients who responded to crizotinib (37), possibly because crizotinib poorly penetrates CNS and is a substrate of P-glycoprotein, a component of the BBB (37).

Surprisingly, combined treatment with crizotinib resulted in remarkable regression of gefitinib-resistant LMC (Fig. 5A). Though *EGFR*-mutant lung cancer cell lines such as PC-9/LMC-GR may acquire intermediate resistance to EGFR-TKIs in LMC, this resistance can be overcome with a low dose of crizotinib. It will be important to develop inhibitors with higher MET selectivity and better potential to penetrate the CNS to better control EGFR-TKI resistance in LMC.

Osimertinib is the third generation EGFR-TKI, which has activity toward EGFR with sensitive *EGFR* mutations (exon 19 deletion and L858R mutation) and the T790M resistance mutation, but spares wild type EGFR (18). This drug highly penetrates the CNS and prevents CNS metastasis in *EGFR*-mutant lung cancer patients (19, 38, 39). However, disease progression in CNS lesions occurs during osimertinib treatment in a population of *EGFR*-mutant lung cancer patients (38). In the LMC model where we inoculated mice with PC-9/LMC-GR cells (Fig 6A), the combined use of crizotinib remarkably regressed LMC with acquired resistance to osimertinib. This model resembles the scenario in *EGFR*-mutant lung cancer patients with LMC that failed first line gefitinib and second line osimertinib therapies, suggesting the efficacy of combined treatment using crizotinib and osimertinib as the third line therapy. MET amplification has been reported in ~10% of extracranial tumor specimens obtained from *EGFR*-mutant lung cancer patients who acquired resistance to EGFR-TKIs (12). It is essential to investigate *MET* copy number gain in LMC cancer cells of *EGFR*-mutant lung cancer patients after the acquisition of resistance to EGFR-TKIs including osimertinib.

In summary, we have demonstrated that EGFR-T790M mutations are less frequent in LMC than extra-CNS tumors obtained from *EGFR*-mutant lung cancer patients who acquired resistance to the EGFR-TKIs gefitinib and erlotinib. Utilizing the gefitinib-resistant LMC model with *EGFR*-mutant lung cancer cells, we have shown that MET activation associated with *MET* copy number gain is involved in acquired gefitinib resistance in LMC. Furthermore, combined treatment with crizotinib

remarkably regressed LMC with acquired resistance to gefitinib and osimertinib. These findings suggest that MET inhibition may be a promising strategy for overcoming acquired resistance to EGFR-TKIs in LMC.

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Figure legends

Figure 1. *EGFR*-T790M mutation was less frequent in *EGFR*-mutant lung cancer patients with LMC. Of 622 samples from 599 NSCLC patients, 32 samples from 28 patients obtained by re-biopsy after failure of EGFR-TKI treatment were confirmed to have *EGFR* mutations. Twelve samples were obtained from cerebrospinal fluid (CSF) of patients with LMC. Twenty samples were obtained from tumor lesions extra-CNS, such as lung metastases and malignant pleural effusions. Four patients provided both LMC and extra-CNS samples. Samples were evaluated for *EGFR* mutations as described in the Materials and Methods.

Figure 2. *EGFR*-T790M mutations were less frequent in the LMC model with acquired resistance to gefitinib. A. PC-9/ffluc cells were inoculated into the leptomeningeal space of SHO-SCID mice (N=4). Mice were administered daily oral treatment with gefitinib (25 mg/kg) from day 5 until day 54. **B.** PC-9/ffluc cells were inoculated subcutaneously into SHO-SCID mice (N = 4). Mice were administered daily oral treatment with gefitinib (25 mg/kg) from day 8 until day 56. C. Tumor samples were harvested from mice with acquired gefitinib resistance, and samples were evaluated for *EGFR* mutations as described in the Materials and Methods.

Figure 3. PC-9/LMC-GR cells, established from LMC mice with acquired gefitinib resistance, showed intermediate resistance to gefitinib *in vitro*.

(A) PC-9/ffluc and PC-9/LMC-GR cells (2×10^3 cells/well) were incubated with various concentrations of gefitinib for 72 hours. Cell viability was determined by MTT assay. Bars represent SD of quadruplicate cultures. Cell lysates were obtained from PC-9/ffluc and PC-9/LMC-GR cells, and analyzed by a receptor tyrosine kinase array kit (**B**) and immunoblot with antibodies toward the

indicated molecules (**C**). (**D**) *MET* copy number determined by FISH. Red signal indicates *MET*, and green signal indicates *CEP7* (control). *MET* was amplified in PC-9/LMC-GR but not in PC-9/ffluc cells. (**E**) *MET* copy number was analyzed by Taqman gene copy number assay. The human squamous cell lung cancer cell line EBC-1 and the human fetal lung fibroblast cell line MRC-5 were used as positive and negative controls, respectively. Error bars represent SD of triplicate independent experiments. **(F)** PC-9/ffluc and PC-9/LMC-GR cells (2×10^3 cells / well) were incubated with various concentrations of osimertinib for 72 hours. Cell viability was determined by MTT assay. Bars represent SD of quadruplicate cultures. Data shown are representative of three independent experiments with similar results.

Figure 4. PC-9/LMC-GR cells were resensitized to gefitinib by MET inhibition. A. PC-9/LMC-GR cells (2×10^3 cells/well) were incubated with various concentrations of gefitinib with or without crizotinib (1 μmol/L) or golvatinib (1 μmol/L) for 72 hours. Then, cell viability was determined by an MTT assay. Bars represent SD of triplicate cultures. **B.** PC-9/LMC-GR cells (2×10^5 cells/well) were incubated in 6-well plates with various concentrations of gefitinib with or without crizotinib (1 μmol/L) or golvatinib (1 μmol/L) for 1 hour. Cell lysate were obtained and subjected to immunoblotting with antibodies toward the indicated molecules. **C.** PC-9/LMC-GR cells were transfected with or without siRNA against MET (si-MET) or control siRNA (si-Scr). Cells were cultured in the presence or absence of gefitinib (0.3 μmol/L) for 3 days. The cell lysates were then harvested and immunoblotted with antibodies against the indicated molecules. **D.** Cells from C were further incubated with or without gefitinib (0.3 μmol/L) for 72 hours and cell viability was determined by MTT assay. The data shown are representative of three independent experiments with similar results.

Figure 5. Combined use of crizotinib overcame acquired resistance to gefitinib in LMC models. **A.** PC-9/ffluc cells (1×10^4) were inoculated into the leptomeningeal space of SHO-SCID mice (N=15). Gefitinib was orally administered daily (25 mg/kg) from day 5 to day 52. Then, mice were randomized into 3 groups (N=5 per group) and treated daily with crizotinib (50 mg/kg) alone, gefitinib (25 mg/kg) alone, or gefitinib (25 mg/kg) plus crizotinib (50 mg/kg). **B.** Representative images of mice are shown.

Figure 6. Combined treatment with crizotinib overcame acquired resistance to osimertinib in LMC models. A. PC-9/LMC-GR cells (1×10^4) were inoculated into the leptomeningeal space of SHO-SCID mice. Daily oral treatment with or without gefitinib (25 mg/kg) or osimertinib (25 mg/kg) was administered starting on day 8 (N=5 per group). In the osimertinib-treated group, mice were treated daily with osimertinib (25 mg/kg) plus crizotinib (50 mg/kg) starting on day 48. Bars represent SDs. **B.** Representative images of mice are shown.

Fig 1

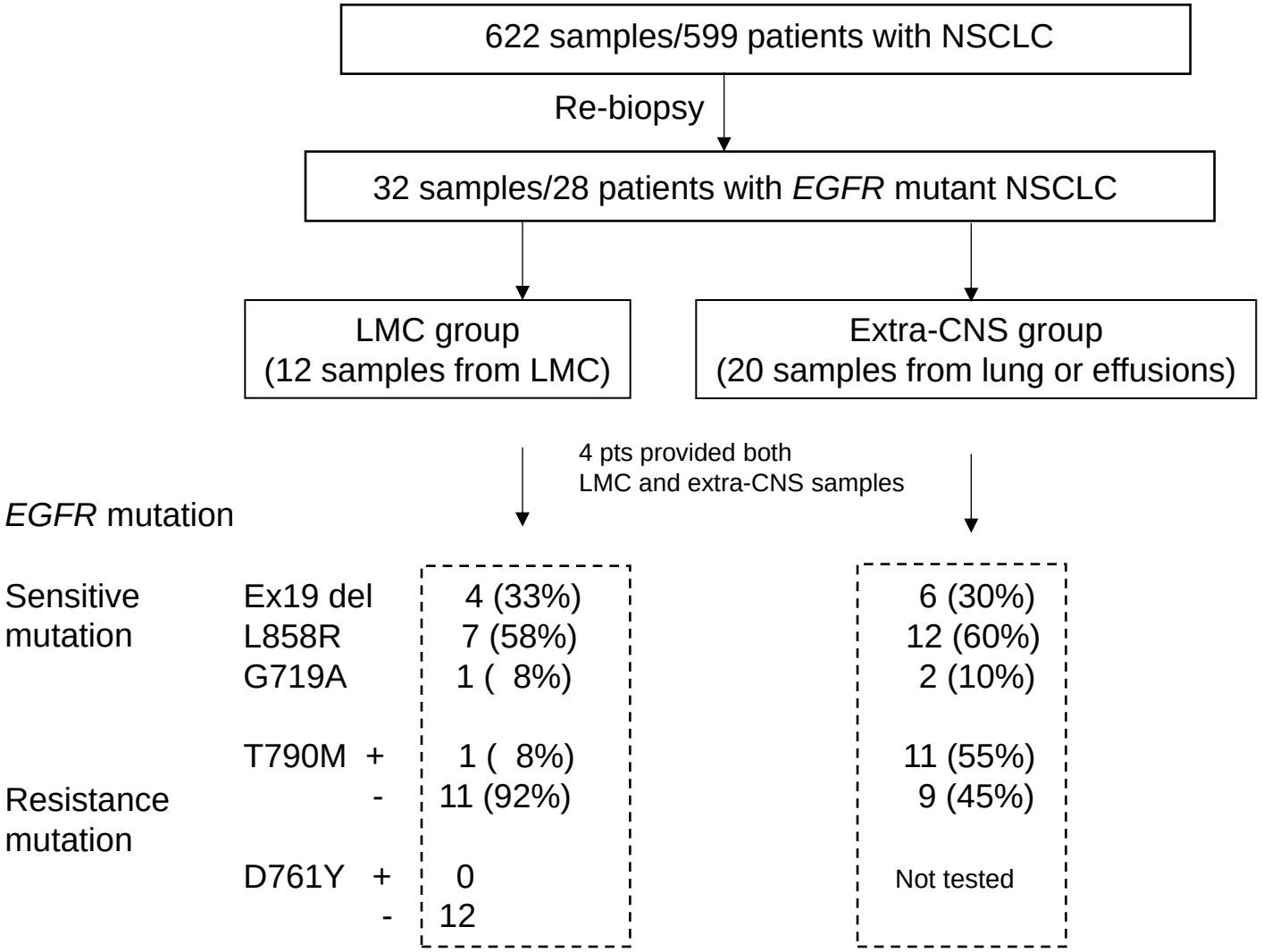
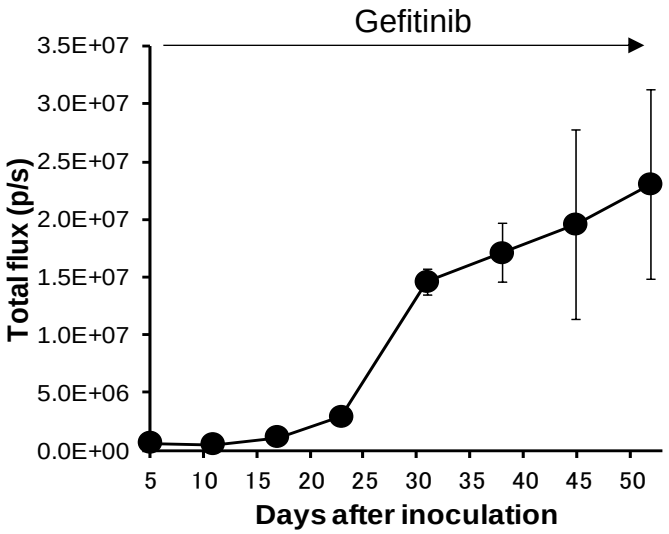
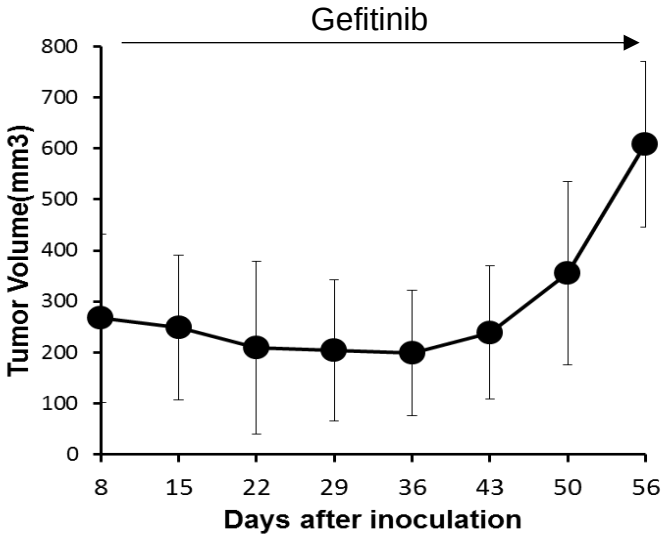

622 samples/599 patients with NSCLC
Re-biopsy
32 samples/28 patients with *EGFR* mutant NSCLC
LMC group
(12 samples from LMC)
Extra-CNS group
(20 samples from lung or effusions)
4 pts provided both
LMC and extra-CNS samples
EGFR mutation
Sensitive mutation
Ex19 del
L858R
G719A
4 (33%)
7 (58%)
1 (8%)
6 (30%)
12 (60%)
2 (10%)
Resistance mutation
T790M +
-
1 (8%)
11 (92%)
11 (55%)
9 (45%)
D761Y +
-
0
12
Not tested

Fig 2

A. LMC model



B. Subcutaneous tumor model



C. *EGFR* mutation status

EGFR mutation	LMC model	Subcutaneous tumor model
Exon 19 deletion	4/4	4/4
T790M	0/4	4/4
D761Y	0/4	0/4

Fig 3

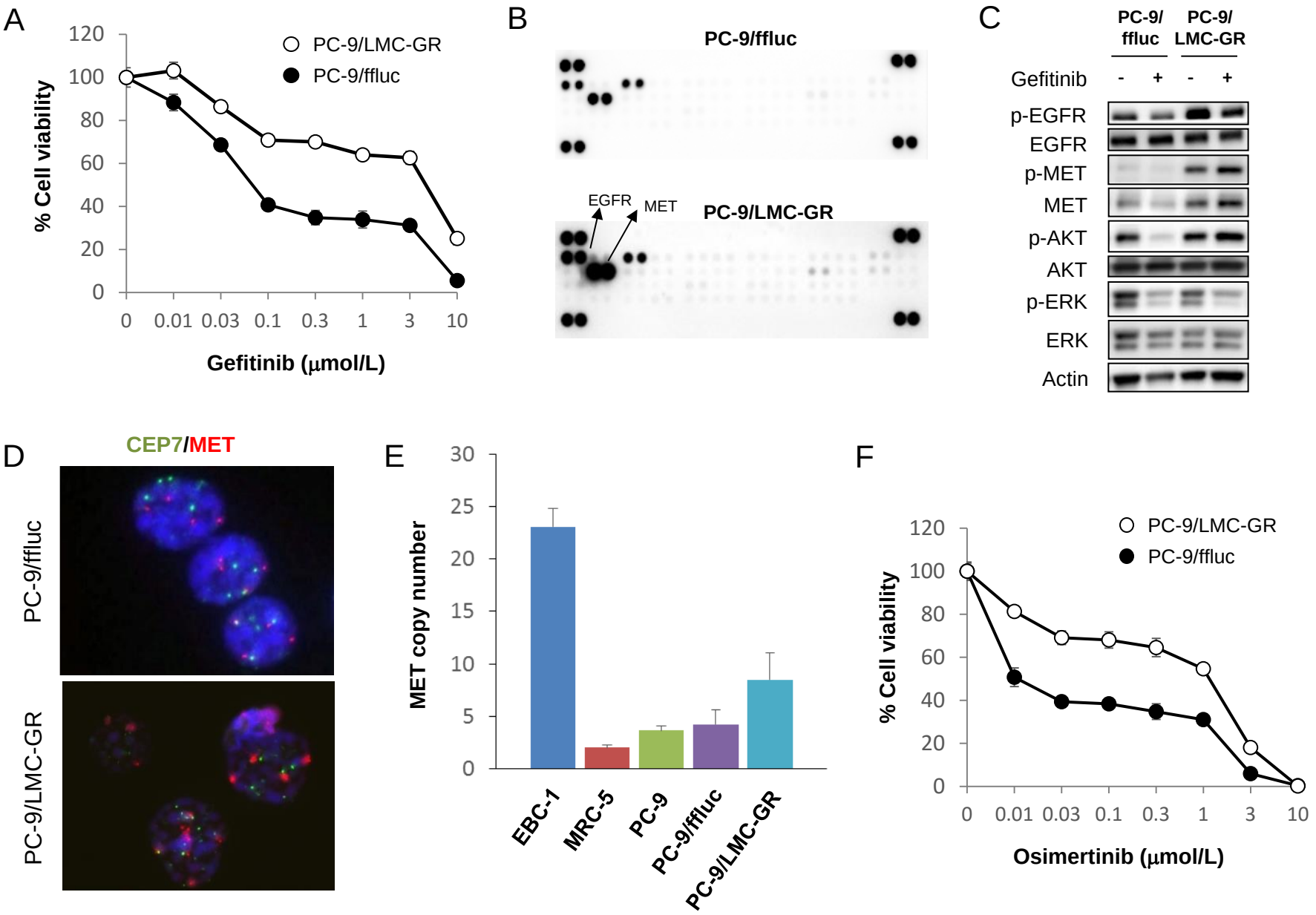

A
120
100
80
60
40
20
0
% Cell viability
PC-9/LMC-GR
PC-9/ffluc
0 0.01 0.03 0.1 0.3 1 3 10
Gefitinib (μmol/L)
B
PC-9/ffluc
EGFR
MET
PC-9/LMC-GR
C
PC-9/ ffluc
PC-9/ LMC-GR
Gefitinib - + - +
p-EGFR
EGFR
p-MET
MET
p-AKT
AKT
p-ERK
ERK
Actin
D
CEP7/MET
PC-9/ffluc
PC-9/LMC-GR
E
30
25
20
15
10
5
0
MET copy number
EBC-1
MRC-5
PC-9
PC-9/ffluc
PC-9/LMC-GR
F
120
100
80
60
40
20
0
% Cell viability
PC-9/LMC-GR
PC-9/ffluc
0 0.01 0.03 0.1 0.3 1 3 10
Osimertinib (μmol/L)

Fig 4

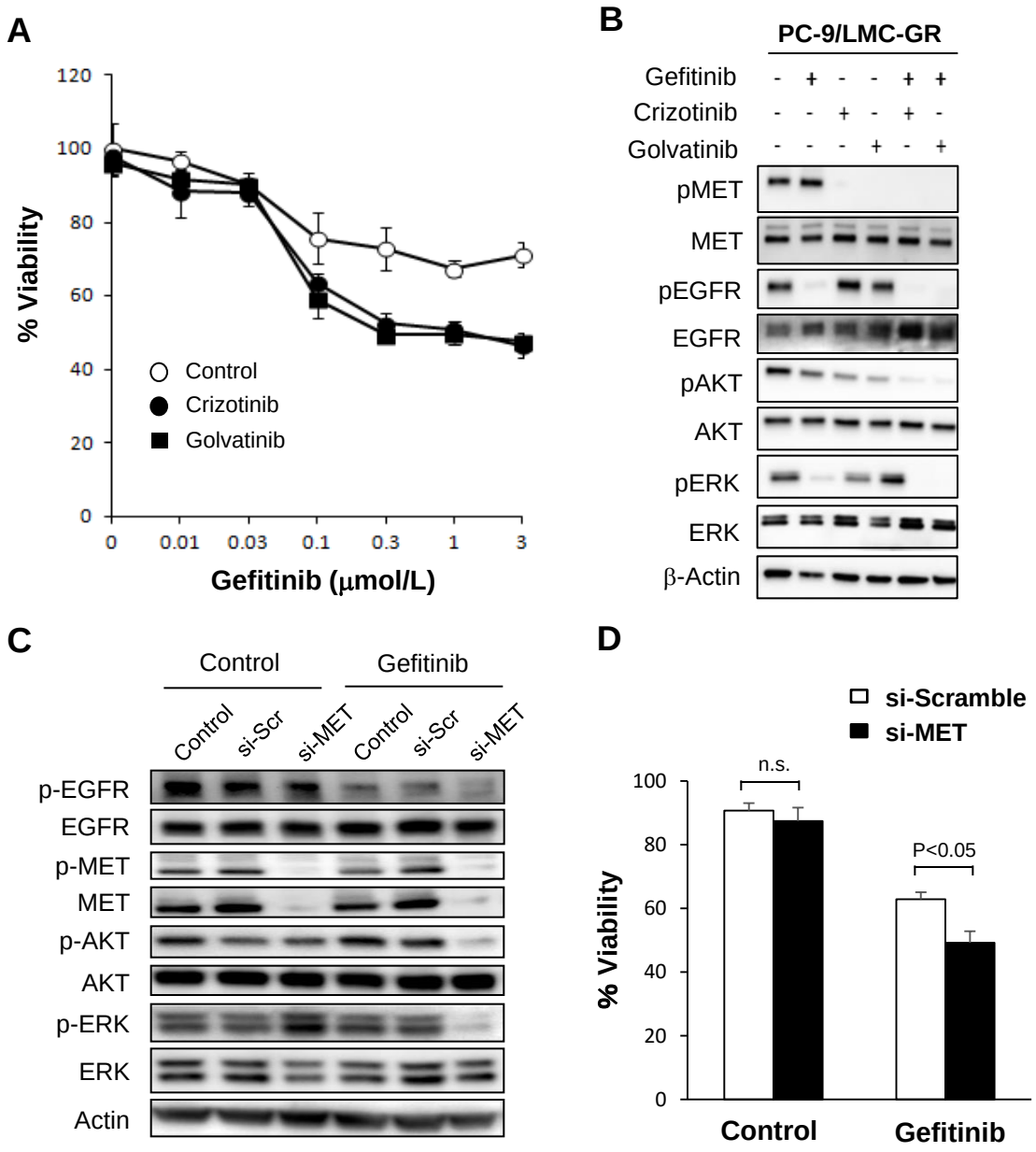

A
120
100
80
60
40
20
0
% Viability
Control
Crizotinib
Golvatinib
0
0.01
0.03
0.1
0.3
1
3
Gefitinib (μmol/L)
B
PC-9/LMC-GR
Gefitinib - + - - + +
Crizotinib - - + - + -
Golvatinib - - - + - +
pMET
MET
pEGFR
EGFR
pAKT
AKT
pERK
ERK
β-Actin
C
Control
Gefitinib
Control
si-Scr
si-MET
Control
si-Scr
si-MET
p-EGFR
EGFR
p-MET
MET
p-AKT
AKT
p-ERK
ERK
Actin
D
si-Scramble
si-MET
n.s.
P<0.05
100
80
60
40
20
0
% Viability
Control
Gefitinib

Fig 5

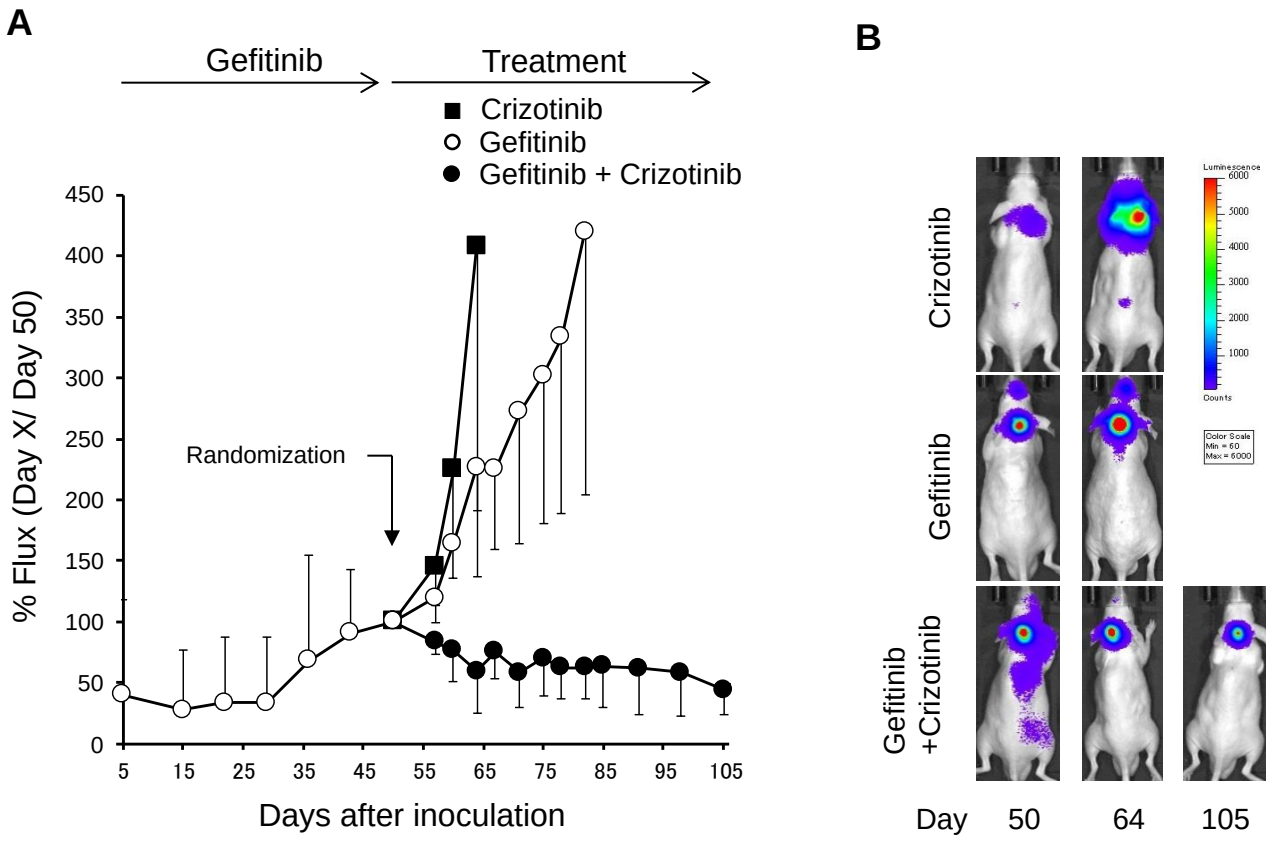
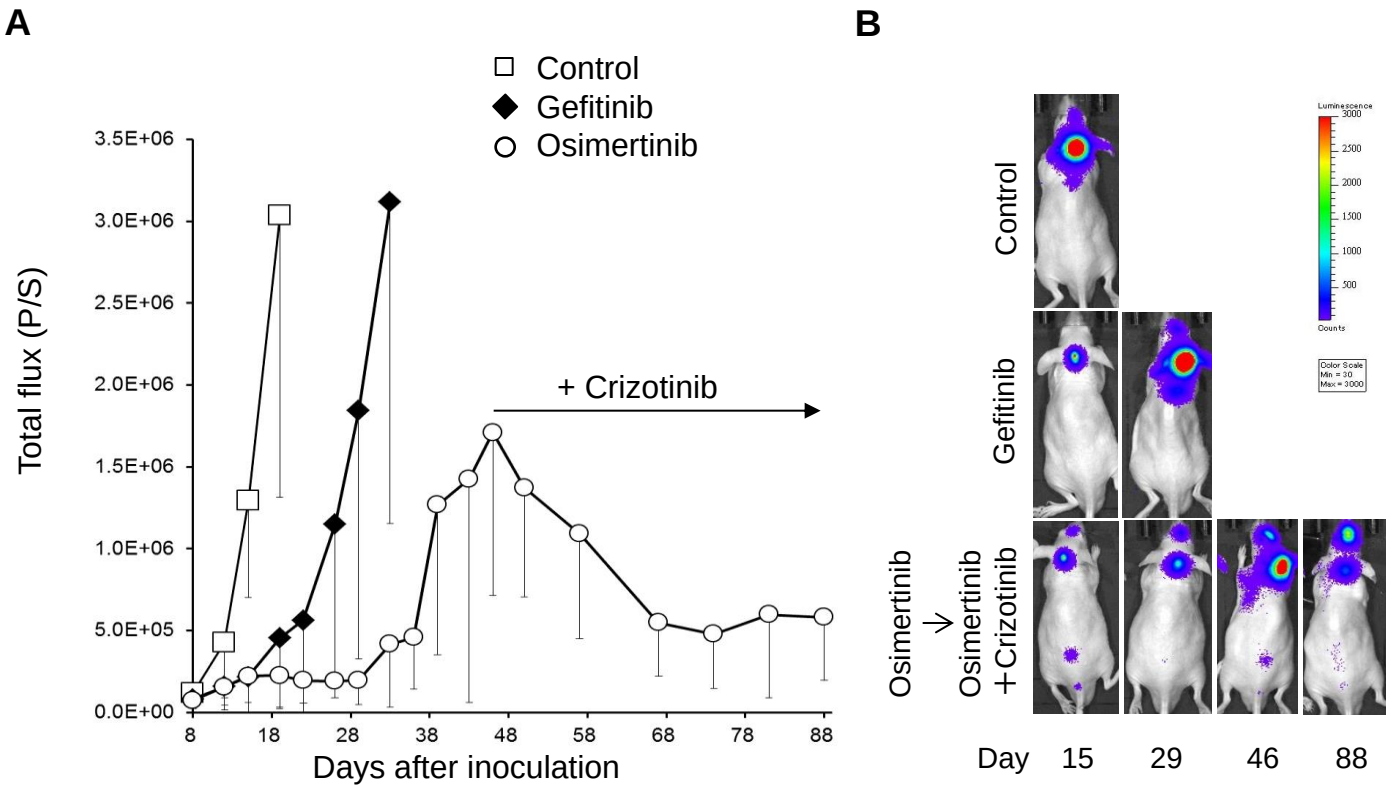

A
Gefitinib
Treatment
Crizotinib
Gefitinib
Gefitinib + Crizotinib
% Flux (Day X/ Day 50)
Randomization
Days after inoculation
B
Crizotinib
Gefitinib
Gefitinib +Crizotinib
Day 50 64 105
Luminescence
Counts
Color Scale
Min = 60
Max = 6000

Fig 6


A
Control
Gefitinib
Osimertinib
Total flux (P/S)
3.5E+06
3.0E+06
2.5E+06
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
+ Crizotinib
8
18
28
38
48
58
68
78
88
Days after inoculation
B
Control
Gefitinib
Osimertinib
Osimertinib
+Crizotinib
Day
15
29
46
88