

Title

EGFR-TKI acquired resistance in lung cancers harboring EGFR mutations in immunocompetent C57BL/6J mice

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Word count: 3653

Figures: 4

Tables: 0

Supplemental figures: 4

Supplemental table: 0

Abstract

Objectives

Lung cancers harboring epidermal growth factor receptor (EGFR) mutations inevitably develop resistance to EGFR tyrosine-kinase inhibitors (EGFR-TKIs). Therefore, we sought to establish clinically relevant lung-cancer mouse models to achieve deep remission of cancers.

Materials and Methods

We previously established two transgenic lung-cancer mouse models harboring human EGFR exon 21 L858R substitution (hLR) and mouse Egfr exon 19 deletion (mDEL) in the C57BL/6J background. Lung tumors from these two transgenic mouse strains were transplanted subcutaneously into BALB/c-nunu mice or C57BL/6J mice.

Results

The transplanted tumors developed the ability to grow on the subcutaneous tissue, peritoneum, or lung of C57BL/6J mice. While hLR tumors could grow only in C57BL/6J mice carrying the transgene, mDEL tumors could grow in wild-type C57BL/6J mice. The tumors maintained EGFR-dependency, and, thus, the EGFR-TKI gefitinib inhibited tumor growth; however, similar to human lung cancers, hLR and mDEL tumors acquired resistance in 60 and 200 days, respectively, following gefitinib administration. Secondary EGFR T790M mutation in hLR tumors and secondary Egfr T792I mutation in mDEL tumors developed; however, no MET activation was detected. Accordingly, the third-generation EGFR-TKI osimertinib effectively inhibited gefitinib-resistant tumors in vivo. Furthermore, gefitinib-resistant tumors developed resistance to osimertinib in 100 days.

Conclusion

These syngeneic lung-cancer mouse models harboring EGFR mutations are suitable for

studying the drug-resistance mechanisms and the role of the tumor microenvironment. Further investigation with these mouse models is warranted for developing next-generation treatment strategies for lung cancer.

Key words: acquired resistance, EGFR mutations, NSCLC, osimertinib, transgenic mice

Introduction

Discovery of oncogenic driver mutations and clinical development of corresponding molecular-targeted inhibitors have led to the application of precision medicine in lung-cancer treatment [1-5]. In patients with non-small cell lung cancer (NSCLC), the most frequently detected mutations are activating mutations of epidermal growth factor receptor (EGFR) [6], and NSCLCs harboring *EGFR* mutations represent one of the most critical subgroups of cancer in clinical practice. In the treatment of NSCLC harboring *EGFR* mutations, EGFR tyrosine-kinase inhibitors (EGFR-TKIs) are highly effective, but drug-resistance inevitably develops in the tumors in ~12–18 months [7-10]. Therefore, to achieve deeper and longer remission, an alternative treatment strategy is required for lung cancers harboring *EGFR* mutations.

To develop highly effective treatment strategies, EGFR-TKI-resistant clinical samples have been widely examined and multiple resistance mechanisms have been discovered, such as drug-resistant *EGFR* T790M mutation, MET amplification, HGF overexpression, ERBB2 amplification, BRAF mutation, and transformation to other types of cancer [11-

16]. Based on these translational-research studies, a third-generation EGFR-TKI, osimertinib, was developed and approved for lung tumors carrying *EGFR* T790M mutation. Subsequently, several bench-to-bedside clinical trials were conducted. For example, gefitinib or osimertinib combined with the MET inhibitor savolitinib or capmatinib showed a beneficial effect in lung cancers that acquired resistance to EGFR-TKIs [17-19]. Moreover, we developed two combination therapies, gefitinib plus bevacizumab or afatinib plus bevacizumab [20, 21], based on the results of preclinical studies conducted using lung-cancer cell lines [22-24]. Collectively, these findings indicate that a translational study is critical for developing an improved rationale for clinical-treatment strategies, and, therefore, it is also crucial to establish clinically relevant lung-cancer models.

Currently, the most common tools used in preclinical studies are cell-line-based models established using human lung-cancer cell lines harboring *EGFR* mutations and xenograft lung-cancer models developed using immunodeficient mice, such as BALB/c-nude mice [8, 22, 24]. However, in these models, it is challenging to validate the interactions among the tumors, the tumor microenvironment, and the immune system, and thus an EGFR-mutant lung-cancer mouse model exhibiting normal immune function would be valuable. A few research groups, including ours, have established genetically engineered lung-cancer mouse models by using C57BL/6 mice featuring a normal immune system [25-28]. By using Clara-cell secretory protein (CCSP) promoter or surfactant protein-C (SP-C) promoter, mutant EGFR was selectively expressed in Clara cells or type II alveolar cells in these mouse models; consequently, EGFR-signaling-dependent adenocarcinoma developed spontaneously in the lungs of these mice, and EGFR-TKIs suppressed the growth of the tumors. However, these mouse lung-cancer models mimic human lung

cancer only to certain extent: the tumors in mice form sequentially in a multifocal manner, which is in contrast to the concurrent, unifocal development of lung cancers in humans. Therefore, with these models, resistance to anticancer drugs, including molecular-targeting agents or immune-checkpoint inhibitors, cannot be readily validated. Here, to develop more clinically relevant lung-cancer mouse models than those currently available, we established novel syngeneic lung-cancer mouse models harboring *EGFR* mutations and also developed EGFR-TKI-resistant lung cancer mouse models.

Materials and Methods

Allotransplantation of mouse lung adenocarcinoma into athymic mice

Lung adenocarcinoma cells were collected from transgenic mice previously established in our laboratory [25, 26]. The transgenic mice harbor human *EGFR* L858R substitution (hLR C57BL/6J) or mouse *Egfr* exon-19 deletion (mDEL C57BL/6J). The SP-C promoter is used here to drive the expression of the mutated genes specifically in type II alveolar cells (Figure S1A, C), and these mice spontaneously develop lung adenocarcinomas (Figure S1B, D). Approximately 1-g samples of lung tumor were minced in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) and dissociated into single-cell suspensions by using a Tumor Dissociation Kit, mouse (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany), and then red blood cells were removed from the suspensions by using the Red Blood Cell Lysis Solution (Miltenyi Biotec). The adenocarcinoma cells were resuspended in 1 mL of PBS plus 1 mL of Matrigel matrix (Corning, New York, NY, USA), and 0.2 mL of this adenocarcinoma suspension was injected into the back of athymic mice (purchased from Charles River Laboratories, Yokohama, Japan). When the tumors that formed became sufficiently large ($>1000 \text{ mm}^3$),

they were dissected in a similar manner and serially transplanted into other athymic mice, and this cycle of passaging was continued. The experimental protocol was approved by the Animal Care and Use Committee, Okayama University, Okayama, Japan (OKU-2017424). The humane endpoints in the protocol (OKU-2017424) were defined as follows: the experiment must be discontinued immediately and the experimental animals must be euthanized when they suffer intolerable pain such as respiratory distress, body weight loss of more than 20%, and decreased food/water intake during the experimental procedure.

Syngeneic lung-cancer mouse models

Female C57BL/6J mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All mice were provided sterilized food and water and were housed in a barrier facility, maintained at an air-conditioned temperature of $22\pm 2^{\circ}\text{C}$ with constant humidity under a 12/12-h light/dark cycle. Adenocarcinoma tumors (1 g) passaged using athymic mice were dissociated into single-cell suspensions as described in the previous section (with the tumor volume depending on the number of mice to be injected with the adenocarcinoma), and after removing red blood cells, the adenocarcinoma cells were resuspended in a 1:1 mixture of PBS and Matrigel matrix; 0.2 mL of these adenocarcinoma-cell suspensions were injected subcutaneously into the back (on both sides) of C57BL/6J mice. When generating the syngeneic mouse model by using hLR cells, rejection was avoided by injecting the cell suspensions into transgenic mice harboring the hLR mutation. When the average tumor volume reached approximately 500 mm^3 , the mice were randomly assigned to groups (3 mice/group) that were treated with vehicle, gefitinib (50 mg/kg/day), or osimertinib (5 mg/kg/day). The vehicle and drugs

were administered once daily, five times per week, by gavage. Tumor volume ($\text{width}^2 \times \text{length}/2$) was determined periodically.

The adenocarcinoma suspensions were also injected into the tail vein or peritoneal cavity of C57BL/6J mice to generate lung-metastasis or peritoneal-metastasis models.

Direct sequencing of EGFR gene

Genomic DNA was isolated from tumors by using a QIAamp DNA Mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol, and direct sequencing for mutation detection was performed using these primers: for human *EGFR* exon-21 point mutation: forward, 5'-CAGATCGCAAAGGGCATGAACT-3', reverse, 5'-CCAGACATCACTCTGGTGGGTATA-3'; for mouse *Egfr* exon-19 deletion: forward, 5'-TTGGCACAGTGTATAAGGGTCTCT-3', reverse, 5'-CATCACATAGGCTTCGTCAAGGATT-3'.

RNA was extracted from cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Direct sequencing for mutation detection was performed using these primers: for human *EGFR* exon-20: forward, 5'-GGCACGGTGTATAAGGGACTC-3', reverse, 5'-TTTGCCTCCTTCTGCATGGT-3'; for mouse *Egfr* exon-20: forward, 5'-TGGCACAGTGTATAAGGGTCTC-3', reverse, 5'-GGCCAGCCCAAATCTGTGA-3'.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks generated using the tumor samples were sectioned at a thickness of 5 μm , and the sections were placed on glass slides and

deparaffinized in Hemo-De (FALMA, Tokyo, Japan) and graded alcohol. For antigen retrieval, sections were incubated in 10 mmol/L sodium citrate buffer, pH 6.0, for 10 min in a 95°C water bath, after which the sections were incubated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. The slides were rinsed with TBS containing 0.1% Tween-20, and the sections were blocked with goat serum for 60 min. Subsequently, the sections were incubated with an anti-EGFR antibody (SC-03, Santa Cruz Biotechnology, Dallas, TX, USA; or #71655, Cell Signaling Technology, Danvers, MA, USA), anti-SP-C antibody (ab90716, Abcam, Cambridge, UK), or anti-CD8 alpha antibody (ab217344, Abcam, Cambridge, UK) overnight at 4°C, and then with EnVision+ System-labeled polymer-horseradish peroxidase anti-rabbit antibody (DakoCytomation, Glostrup, Denmark) for 20 min. Lastly, the sections were counterstained with hematoxylin.

Western blotting and receptor tyrosine kinase (RTK) array

Mice were exposed to drug treatments when tumors were ~500 mm³ in size, and tumor tissue was collected at 4 h after drug administration and immediately frozen in liquid nitrogen. The frozen tissue was pulverized using a cryo-press and incubated for 30 min in lysis buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerol phosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate-containing protease-inhibitor tablets (Roche Applied Sciences, Mannheim, Germany)]. The suspension was centrifuged at 15000 rpm for 30 min at 4°C. Proteins were electrophoretically separated on polyacrylamide gels, transferred onto nitrocellulose membranes, probed with specific antibodies, and detected using Enhanced Chemiluminescence Plus (GE Healthcare Biosciences, Piscataway, NJ,

USA); antibodies against the following targets were obtained from Cell Signaling Technology: phospho-EGFR (#3777), EGFR (#2232, #71655), phospho-AKT (#9271), AKT (#9272), phospho-ERK (#9101), ERK (#9102), and GAPDH (#2118). The anti-MET antibody was purchased from Abcam (ab51067). A Mouse Phospho-RTK Array Kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions. Bands and dots were detected using an ImageQuant LAS-4000 imager (GE Healthcare Biosciences).

Results

Mouse lung tumors harboring *EGFR* mutations could be engrafted onto subcutaneous tissue, peritoneum, and lung of C57BL/6J mice

We previously generated two strains of transgenic lung-cancer mouse models harboring hLR or mDEL; multiple lung tumors developed spontaneously from type II alveolar cells in the bilateral lungs of these mouse models generated using C57BL/6J mice (Figure S1A-D)[25, 26]. To establish syngeneic lung-cancer mouse models, we transplanted the lung-tumor cells from these mice into other mice. First, the mDEL tumors were dissected and transplanted into the subcutaneous tissue of wild-type C57BL/6J mice. However, the tumors could be engrafted once, and then underwent necrosis, and the wild-type C57BL/6J mice bearing the mDEL tumors died within a few weeks. No metastatic lesions were detected in any of the organs (e.g., lung, liver) in the mice. The tumor could be engrafted into other wild-type C57BL/6J mice, if they were serially transplanted before the mice died. However, the handling was complicated, and the model was too unstable for long term maintenance. Therefore, we next transplanted the hLR or mDEL tumors into athymic mice. The engrafted tumors maintained their original sizes for a few weeks

and then started to grow slowly in the athymic mice (Figure 1A), and the tumors grew to an adequate size for passage at 4 and 10 months after transplantation of the hLR and mDEL adenocarcinomas, respectively. Both types of tumors were serially transplanted onto the back of other athymic mice, and after several passages, the tumors developed the capacity for being transplanted serially once monthly into athymic mice. Immunohistochemical staining confirmed that the tumors expressed SP-C and EGFR (Figure 1B, D), and direct sequencing confirmed that *EGFR* mutations were preserved in the tumors (Figure 1C, E). Subsequently, we re-tested whether these tumors could grow on the back of wild-type C57BL/6J mice. The mDEL tumors could be successfully engrafted, and approximately 4 weeks later, the tumor growth was sufficient for serial transplantation onto the back of wild-type C57BL/6J mice. By contrast, the hLR tumors were rejected on wild-type C57BL/6J mice, although, intriguingly, the tumors could be successfully engrafted onto and could grow on subcutaneous tissue of hLR C57BL/6J mice (which harbor human *EGFR* L858R mutation). CD8-positive lymphocytes infiltrated the mDEL tumors in wild-type C57BL/6J mice and hLR tumors in hLR C57BL/6J mice (Figure S1E, F).

Next, we injected the hLR and mDEL tumors separately into the peritoneum and tested for tumor growth; as expected based on the aforementioned results, the two types of tumor cells grew in the peritoneum in wild-type C57BL/6J mice and hLR C57BL/6J mice, respectively (Figure S1G, H). We also assessed whether the mDEL tumor cells exhibited the capacity for hematogenous dissemination into the lung when they were injected into the tail vein in wild-type C57BL/6J mice: computed tomography scanning revealed that these tumor cells formed multiple metastatic nodules in the bilateral lungs (Figure S1I). However, in macroscopic and microscopic examination, we detected no engrafted tumors

in the liver or other organs. Thus, we newly established three types of syngeneic EGFR-mutant lung-cancer mouse models: a subcutaneous tumor model, a metastatic peritoneal tumor model, and a metastatic lung tumor model. These tumors presented similar morphological features (Figures 1B, D and S1B, D, G-I).

Effect of EGFR-TKIs on engrafted lung tumors

We next assessed whether the growth of engrafted tumors retained a dependence on EGFR signaling, and we observed, as expected, that the EGFR-TKI gefitinib (50 mg/kg, 5 days/week p.o.) markedly inhibited the growth of hLR and mDEL tumors in subcutaneous tumor models (Figure 2A, C). We also tested the effect of another EGFR-TKI, afatinib (10 mg/kg, 5 days/week p.o.), and found that this inhibitor, like gefitinib, strongly inhibited tumor growth in the mDEL subcutaneous model (Figure S2A). Western blotting further revealed that gefitinib treatment diminished EGFR phosphorylation in hLR and mDEL tumors in a dose-dependent manner (Figure 2B, D). Next, the EGFR-TKI effect was assessed in a lung-metastasis model by using mDEL tumors: gefitinib administration for 2 weeks substantially inhibited the growth of these lung metastatic tumors, but the tumors regrew quickly, by 2 weeks, after cessation of gefitinib treatment (Figure S2B). Collectively, these results suggested that the tumorigenic growth of both hLR and mDEL tumor cells still depended on EGFR signaling in vivo.

Gefitinib-resistant mouse lung tumors harboring *EGFR* mutations

To investigate the EGFR-TKI-resistance mechanism and develop alternative treatment strategies for lung cancers harboring *EGFR* mutations, we sought to establish EGFR-TKI-resistant lung-cancer models. We engrafted hLR or mDEL tumors on the back of

nude mice and administered gefitinib (50 mg/kg, 5 days/week p.o.) until the tumors acquired drug-resistance. We defined acquired resistance as an increase in tumor size under the treatment, with the achieved volume being close to the starting tumor volume. Gefitinib treatment effectively inhibited the tumors initially, but by approximately 60 and 200 days, respectively, after initiation of gefitinib administration, the hLR and mDEL tumors regrew under the gefitinib treatment (Figure 3A, D).

The gefitinib-resistant tumors were next transplanted into other nude mice, and the tumors were treated with gefitinib for 2 weeks to assess whether the resistance was maintained after the transplantation. As expected, both types of the gefitinib-resistant tumors retained their drug-resistance: treatment with gefitinib (50 mg/kg, 5 days/week p.o.) did not inhibit the growth of the resistant hLR or mDEL tumors *in vivo*, although the drug treatment effectively suppressed tumor growth in mice bearing parental hLR or mDEL tumors (Figure 3B, E). Intriguingly, direct sequencing revealed that the gefitinib-resistant tumors had developed clinically relevant drug-resistance mutations: the gefitinib-resistant hLR tumors harbored a secondary *EGFR* T790M (c.2369C>T) mutation and the gefitinib-resistant mDEL tumors harbored a secondary *Egfr* T792I (c.2375C>T) mutation (Figure 3C, F); direct sequencing of the target complementary strand revealed the same mutation as in the reading strand (Figure S3A, D). Digital PCR detected *EGFR* L858R and *EGFR* T790M in cell free DNA from the cardiac blood of athymic mice bearing gefitinib-resistant hLR tumors (Figure S4). Notably, neither the hLR nor the mDEL parental tumors harbored these mutations at the same position (Figure 3C, F). Furthermore, neither overexpression of MET protein nor activation of MET phosphorylation was detected in the resistant tumors (Figure S3B, C, E, F).

Efficacy of osimertinib toward gefitinib-resistant tumors

Lastly, we evaluated the effect of osimertinib, a third-generation EGFR-TKI, on gefitinib-resistant tumors; osimertinib has been approved for clinical lung tumors harboring a secondary resistant *EGFR* T790M mutation [29]. In accordance with its effect on human lung cancers, osimertinib (5 mg/kg) strongly inhibited the *in vivo* growth of the gefitinib-resistant hLR tumors harboring *EGFR* L858R and T790M mutations, but the tumor growth was not suppressed by gefitinib (Figure 4A). Western blotting results further showed that osimertinib (5 mg/kg) inhibited the phosphorylation of EGFR and the downstream signaling proteins AKT and ERK in the gefitinib-resistant hLR tumors *in vivo*, whereas gefitinib (5 or 50 mg/kg) did not diminish the EGFR phosphorylation level in these tumors (Figure 4B). Similarly, osimertinib (5 mg/kg) markedly inhibited the *in vivo* growth of gefitinib-resistant mDEL tumors harboring *Egfr* exon-19 deletion and T792I mutation, but gefitinib exerted limited effect on the tumor growth (Figure 4C), and whereas osimertinib (5 mg/kg) inhibited the phosphorylation of EGFR, AKT, and ERK in gefitinib-resistant mDEL tumors *in vivo*, gefitinib (5 or 50 mg/kg) only modestly suppressed the phosphorylation of these proteins (Figure 4D). Furthermore, RTK-array analyses revealed that no RTK other than EGFR was activated in either of the gefitinib-resistant tumors (Figure S3C, F). Approximately 100 days after initiation of osimertinib administration, the gefitinib-resistant hLR and gefitinib-resistant mDEL tumors regrew under the osimertinib treatment (Figure 4E, F). Both of the osimertinib-resistant tumors did not develop EGFR C797S mutation.

Discussion

To achieve deep remission and cure for patients with advanced lung cancer, next-

generation treatment strategies must be developed. Here, we established syngeneic lung-cancer mouse models harboring *EGFR* mutations by using immunocompetent C57BL/6J mice; these models present three clinically relevant features: (1) the lung tumors are derived from type II alveolar cells of immunocompetent C57BL/6J mice; (2) the tumor growth depends on EGFR signaling and shows sensitivity to EGFR-TKIs, and the tumors develop secondary resistant *EGFR* mutations [*EGFR* T790M (c. 2369 C>T) or *Egfr* T792I (c. 2375 C>T)] after chronic exposure to gefitinib; and (3) osimertinib inhibits the growth of gefitinib-resistant lung cancers in C57BL/6J mice. Therefore, our lung-cancer mouse models closely reflect the clinical course of human lung cancers [29, 30].

A few research groups have previously established transgenic mice harboring *EGFR* mutations, but our mice offer certain advantages when compared with other models. In the transgenic mice generated by other groups, reverse tetracycline transactivator is used for the Tet system, but this is not the case with our transgenic mice [25-28]. Considering the toxicity of tetracycline [31], our transgenic mice are more suitable than previous mouse models for long-term tumor passage or assessment of the effect of cancer drugs. Additional strengths of our lung-cancer models are the following: First, our models enable the generation of diverse metastasis models, such as models for subcutaneous metastasis, peritoneal metastasis, and multiple pulmonary metastases. Creating various metastasis models is critical because tumor response to a treatment can occasionally differ according to the site of metastasis [32, 33]. Second, these lung tumors can be engrafted on tissues in immunocompetent C57BL/6J mice. We confirmed the infiltration of CD8-positive lymphocytes in the tumors (Figure S1E, F). Recently, the interaction between tumor cells and the tumor microenvironment, including cancer associated fibroblast or tumor associated macrophage, has been shown to be related to the persistence of drug

tolerance [34, 35]. Therefore, our models could serve as an appropriate platform for investigating the function of the tumor microenvironment in the resistance to EGFR-TKIs. Stromal cells or immune cells might play a role in drug-resistance [34-36], and, thus, the tumor microenvironment could represent a potential alternative treatment target. To further explore the role of the tumor microenvironment in the development of resistance to EGFR-TKI, we are currently developing EGFR-TKI resistant tumors in C57BL/6J mice.

A few limitations of this study are the following. With regard to the tumor models, similarities and differences between mice and humans must be considered [37, 38]. Moreover, our lung-cancer models did not acquire spontaneous metastatic ability; thus, the introduced genetic aberration might be insufficient for the development of such a phenotype [38, 39]. If metastatic lung tumors are serially implanted into other mice through injection via the tail vein, the tumors might acquire metastatic capability. We have also attempted to establish mouse cancer cell lines in vitro by using several distinct conditions, but this effort has not yet been successful. Additionally, we did not clarify how tumors acquired the ability to grow stably in C57BL/6J mice after engraftment into athymic mice. Some genetic or epigenetic changes related to immunogenicity might produce immunoediting. Furthermore, hLR tumors could be engrafted only in hLR C57BL/6J but not in wild type C57BL/6J mice; in contrast, mDEL tumors could be engrafted in wild type C57BL/6J mice. The human EGFR protein could have exerted immunogenic effects on the immune system of wild type C57BL/6J mice. One explanation for this outcome might be that the hLR C57BL/6J mice exhibited immune tolerance as human EGFR L858R protein had been present in their lungs since their juvenile years.

Another limitation is that we detected only one mechanism of acquired resistance. Politi et al reported that in *EGFR*-mutated mouse lung cancer developed using the CCSP promoter, multiple resistance mechanisms were detected, including *EGFR* T790M mutation, MET amplification, and KRAS mutation [40]. MET activation was not detected in our models, and several potential explanations exist for our results being distinct from the previous results. First, the CCSP promoter was used in the previous model, and therefore the tumor developed from Clara cells; by contrast, the SP-C promoter was used in our models, and thus the tumors developed from type II alveolar cells. Second, a preclinical study showed that drug dosage or schedule can potentially affect the resistance-development mechanisms in vitro [41]. Whereas Politi et al treated mice with erlotinib (25 mg/kg, 5 days/week for 4 weeks with 4 weeks without drug treatment), we treated our mice with gefitinib (50 mg/kg, 5 days/week with no break in drug treatment). The modified treatment setting might produce multiple resistance mechanisms and liquid biopsy might enable the monitoring of genetic changes in our models.

In conclusion, we established syngeneic EGFR-TKI-resistant lung-cancer mouse models. Although EGFR-TKI treatment is standard care for patients with lung tumors harboring *EGFR* mutations, deep remission or cure is rarely achieved. Our lung-cancer models provide a new platform for studying the biology of drug-tolerant, persistent cancer cells, the mechanisms of development of drug-resistance, and the underlying role of the tumor microenvironment; further investigation with our mouse models is warranted for developing next-generation treatment strategies for lung cancer.

Acknowledgements

We are grateful to Hiromi Nakashima and Kyoko Maeda for technical support. We also thank Dr Takehiro Matsubara (Division of Biobank, Center for Comprehensive

Genomic Medicine, Okayama University Hospital) for analyzing digital PCR, and our laboratory colleagues for the useful discussions. This work was supported by JSPS Grants-in-Aid for Scientific Research (Grant-in-Aid for Young Scientists [B]: KAKEN 16K19454) to K.O. and JSPS Grants-in-Aid for Scientific Research (Scientific Research [B]: KAKEN 15H04830) to K.O. and K.K. This research was also supported by a grant provided by Boehringer-Ingelheim, Japan to K.O.

Disclosure Statement: K.O. received a research grant from Boehringer-Ingelheim, Japan. All other authors declare no conflicts of interest regarding this study.

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Figure legends

Figure 1

Establishment of syngeneic lung-cancer mouse models harboring EGFR mutations.

HE: hematoxylin and eosin; EGFR: epidermal growth factor receptor; SP-C: surfactant protein C; hLR, tumors harboring human *EGFR* L858R mutation; mDEL, tumors harboring mouse *Egfr* exon-19 deletion.

- A. Schema of serial transplantation of a lung-cancer tumor harboring *EGFR* mutation.
- B. Microscopic examination of HE-stained and immunohistochemically stained hLR tumor tissues. Scale bar = 30 μ m.
- C. Direct sequencing of human *EGFR* exon 21 in hLR tumors.
- D. Microscopic examination of HE-stained and immunohistochemically stained mDEL tumor tissues. Scale bar = 30 μ m.
- E. Direct sequencing of mouse *Egfr* exon 19 in mDEL tumors.

Figure 2

Effect of gefitinib on syngeneic lung tumors harboring EGFR mutations.

Tumors harboring human *EGFR* L858R mutation, hLR; tumors harboring mouse *Egfr* exon-19 deletion, mDEL.

- A. Effect of EGFR-TKI gefitinib on hLR tumor growth in syngeneic lung-cancer models; hLR tumors were treated with 50 mg/kg/day gefitinib or vehicle. Error bars, SE.
- B. Inhibitory effect on EGFR signaling pathway in syngeneic tumors was assessed using western blotting; hLR tumors were treated with vehicle or gefitinib (5, 50 mg/kg) for 4 h.

- C. Effect of EGFR-TKI gefitinib on mDEL tumor growth in syngeneic lung-cancer models; mDEL tumors were treated with 50 mg/kg/day gefitinib or vehicle. Error bars, SE.
- D. Inhibitory effect on EGFR signaling pathway in syngeneic tumors was assessed using western blotting; mDEL tumors were treated with vehicle or gefitinib (5, 50 mg/kg) for 4 h.

Figure 3

Establishment of gefitinib-resistant lung tumors harboring secondary *EGFR* mutations.

Tumors harboring human *EGFR* L858R mutation, hLR; tumors harboring mouse *Egfr* exon-19 deletion, mDEL.

- A. Engrafted hLR tumors on subcutaneous tissue of BALB/c-nunu mice acquired resistance to gefitinib at approximately 60 days.
- B. Resistance to gefitinib was maintained in gefitinib-resistant hLR tumors after passage on subcutaneous tissue of other BALB/c-nunu mice.
- C. Direct sequencing results indicating secondary *EGFR* T790M (c.2369C>T) mutation in gefitinib-resistant hLR tumors.
- D. Engrafted mDEL tumors on subcutaneous tissue of BALB/c-nunu mice acquired resistance to gefitinib at approximately 200 days.
- E. Resistance to gefitinib was maintained in gefitinib-resistant mDEL tumors after passage on subcutaneous tissue of other BALB/c-nunu mice.
- F. Direct sequencing results indicating secondary *Egfr* T792I (c.2375C>T) mutation in gefitinib-resistant mDEL tumors.

Figure 4

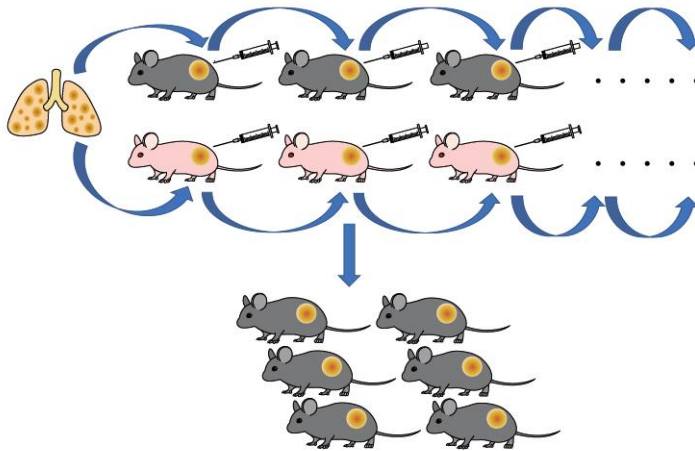
Efficacy of osimertinib in gefitinib-resistant lung tumors harboring secondary *EGFR* mutations.

Tumors harboring human *EGFR* L858R mutation, hLR; tumors harboring mouse *Egfr* exon-19 deletion, mDEL.

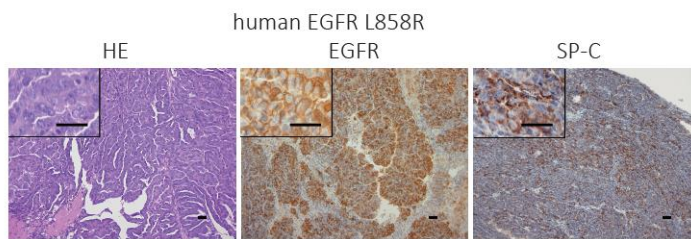
- A. Efficacy of osimertinib toward gefitinib-resistant hLR tumors in subcutaneous tissue of C57BL/6J mice. Gefitinib-resistant hLR tumors were treated with 50 mg/kg/day gefitinib, 5 mg/kg/day osimertinib, or vehicle. Error bars, SE.
- B. Inhibitory effect on EGFR signaling pathway in gefitinib-resistant hLR tumors was assessed using western blotting; gefitinib-resistant tumors were treated with vehicle, gefitinib (5, 50 mg/kg), or osimertinib (5 mg/kg) for 4 h.
- C. Efficacy of osimertinib toward gefitinib-resistant mDEL tumors in subcutaneous tissue of C57BL/6J mice. Gefitinib-resistant mDEL tumors were treated with 50 mg/kg/day gefitinib, 5 mg/kg/day osimertinib, or vehicle. Error bars, SE.
- D. Inhibitory effect on EGFR signaling pathway in gefitinib-resistant mDEL tumors was assessed using western blotting; gefitinib-resistant tumors were treated with vehicle, gefitinib (5, 50 mg/kg), or osimertinib (5 mg/kg) for 4 h.
- E. Engrafted gefitinib-resistant hLR tumors on subcutaneous tissue of BALB/c-nunu mice acquired resistance to osimertinib at approximately 100 days.
- F. Engrafted gefitinib-resistant mDEL tumors on subcutaneous tissue of BALB/c-nunu mice acquired resistance to osimertinib at approximately 100 days.

Figure 1

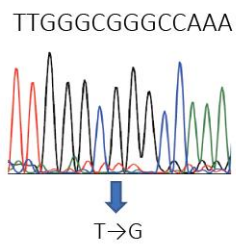
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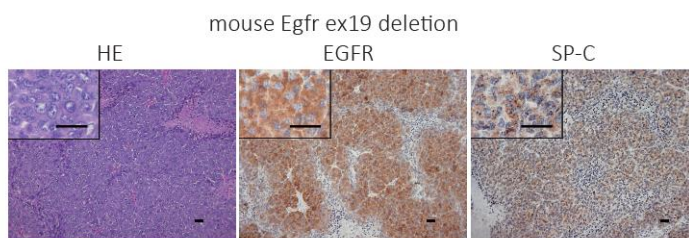
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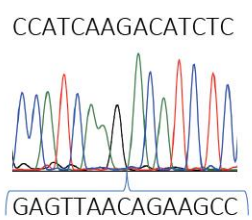


Figure 2

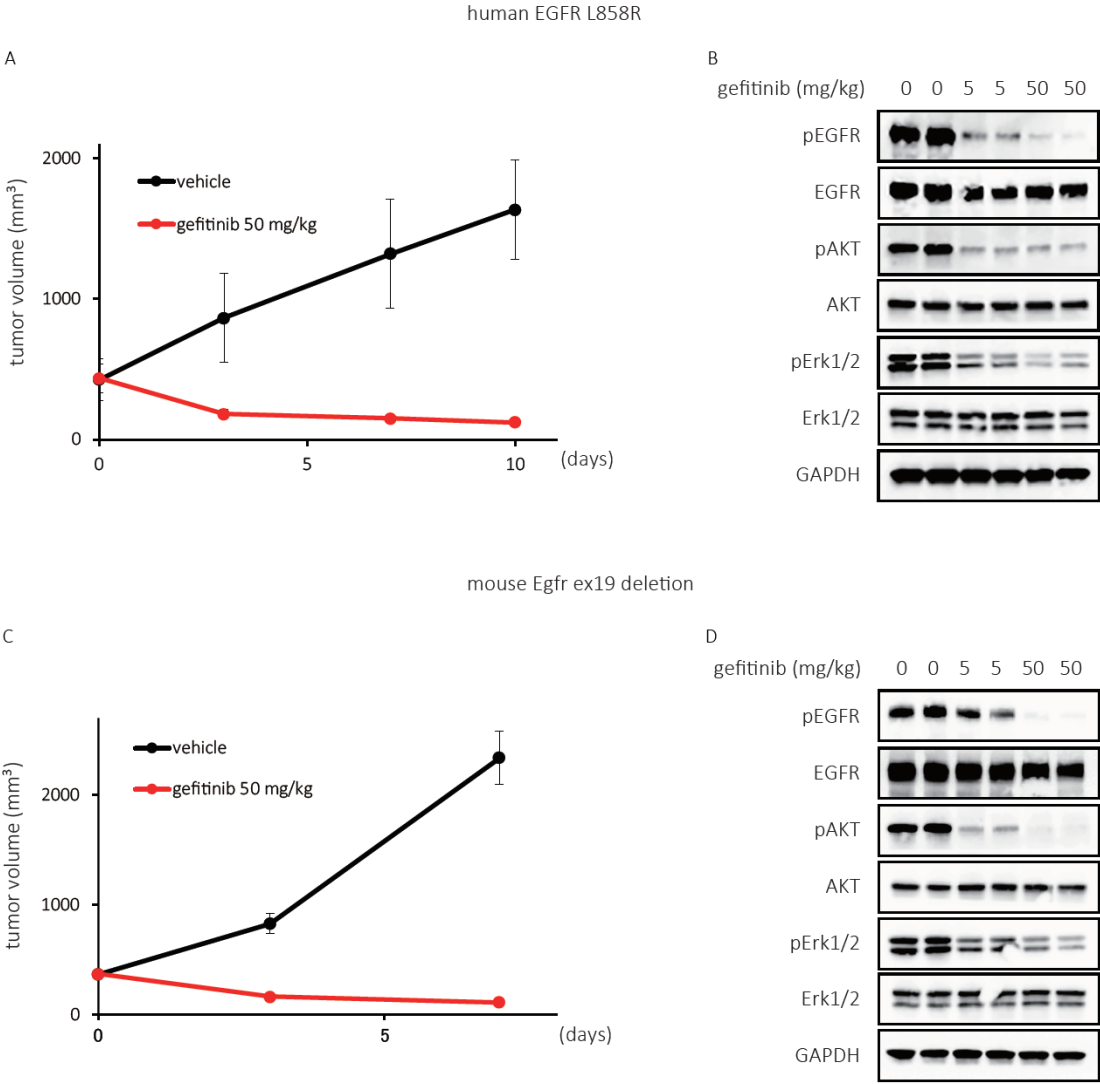


Figure 3

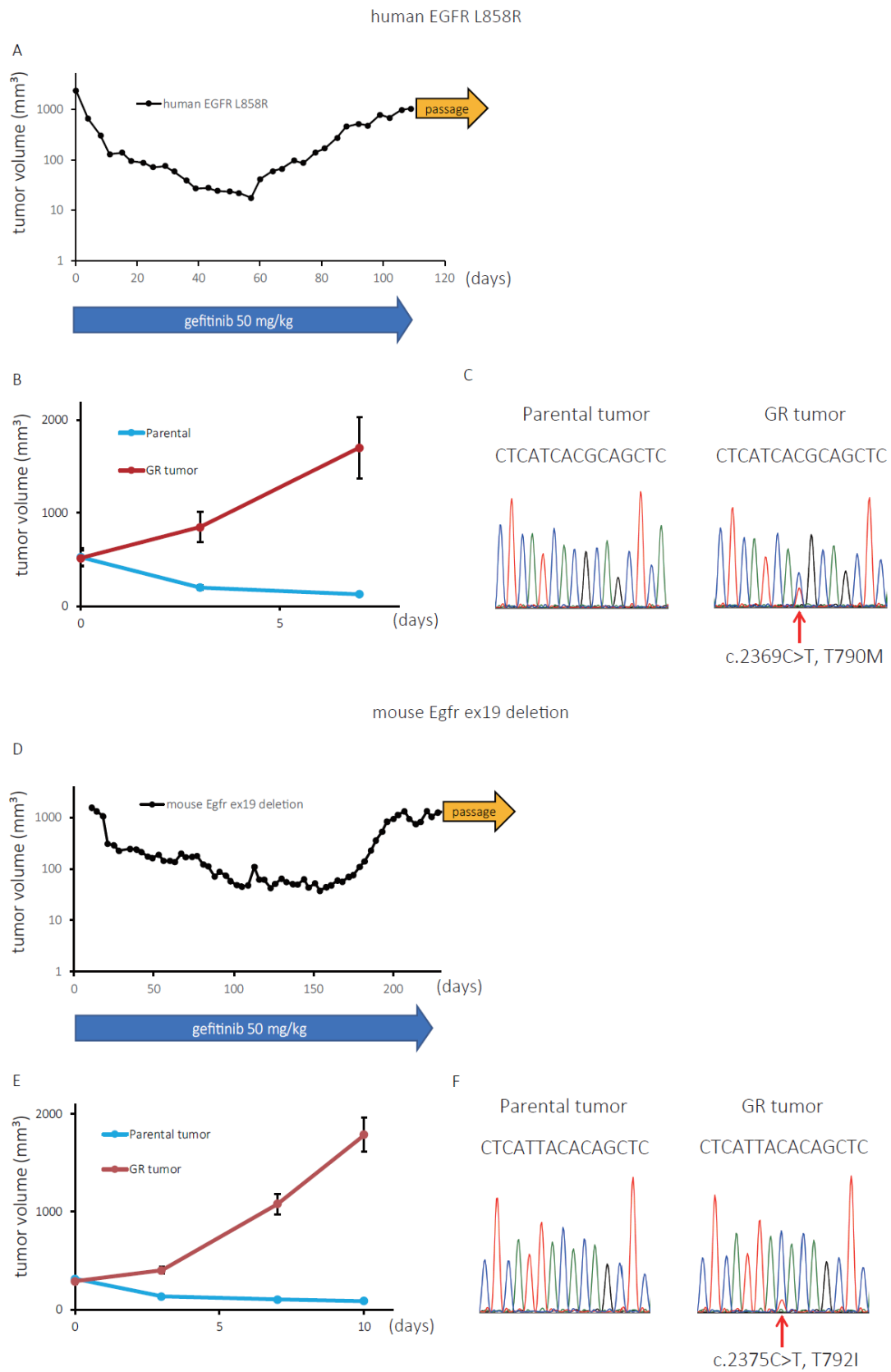
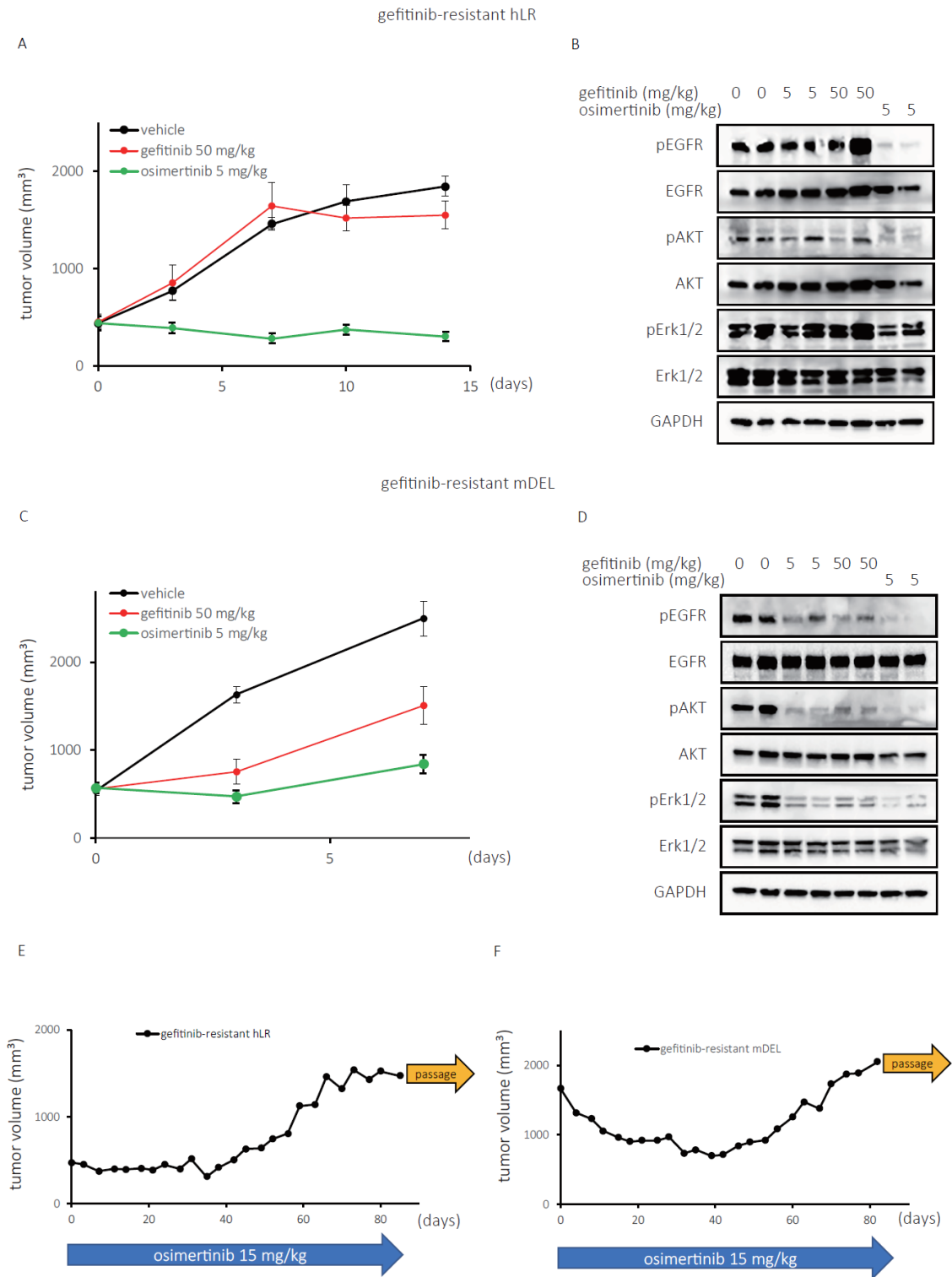


Figure 4



Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the manuscript.

Supplementary materials and methods

Figure S1. Transgenic mice harboring *EGFR* mutations.

Figure S2. Effects of EGFR-TKIs on tumor growth and metastasis in lung-cancer mouse models.

Figure S3. Gefitinib-resistant lung tumors harboring secondary *EGFR* mutations.

Figure S4. Quantification of EGFR L858R, EGFR T790M and wild type EGFR of cell free DNA using droplet digital PCR.

Supplementary materials and methods

Extraction of cell free DNA (cfDNA)

Cardiac blood (200 μ l) was collected from terminally anaesthetized mice into K3-EDTA vacutainers. Blood was centrifuged at 2500 rpm for 10 min, and the plasma supernatant was centrifuged at 1000 $\times g$ for a further 10 min. Blood samples were processed promptly to reduce hemolysis; samples with evidence of hemolysis upon visual inspection were excluded from further analysis. Plasma volumes were adjusted to 200 μ l with PBS before extraction of cfDNA. cfDNA was extracted from a 200- μ l plasma sample using a QIAamp DNA Blood Mini kit (Qiagen) and eluted in 50 μ l TE buffer (10.0 mM Tris-HCl, 0.5 mM EDTA, pH 8.0).

Droplet digital PCR primers and probes

The following primer and probe kits were purchased from Bio-Rad (Hercules, CA, USA); ddPCR Mutation Assay: EGFR p.L858R c.2573T>G (#10049550 Assay ID: dHsaMDV2010019), ddPCR Mutation Assay: EGFR p.T790M, Human (#10049550 Assay ID: dHsaMDV2010021).

Droplet digital PCR assay for EGFR gene mutation detection.

ddPCR was performed at Biobank (Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan) using QX200 Droplet Digital PCR system (Bio-Rad) according to the manufacturer's protocol. The following PCR conditions were used for ddPCR: 1) an initial denaturation step at 95°C for 10 min followed by 2) 45 cycles at 94°C for 30 s, and 3) 45 cycles at 57°C for 1 min. 4) final

enzyme deactivation step at 98°C for 10 min. Each ramp rate was 2°C per second. PCR products were then subjected to analysis by the QX-200 droplet reader and QuantaSoft analysis software (Bio-Rad).

Figure S1

Transgenic mice harboring *EGFR* mutations.

HE: hematoxylin and eosin; EGFR: epidermal growth factor receptor; SP-C: surfactant protein C; hLR, tumors harboring human *EGFR* L858R mutation; mDEL, tumors harboring mouse *Egfr*^{de1748-752}.

- A. Constructs used to generate transgenic mice harboring human *EGFR* L858R mutation and mouse *Egfr* exon-19 deletion. Human *EGFR*^{L858R} was cloned between SP-C promoter and SV40 small T intron¹.
- B. Microscopic examination of HE-stained lung tissue of transgenic mice harboring human *EGFR* L858R mutation. Scale bar = 30 μ m.
- C. Constructs used to generate transgenic mice harboring mouse *Egfr* exon-19 deletion. Mouse *Egfr*^{de1748-752} was cloned between SP-C promoter and SV40 small T intron².
- D. Microscopic examination of HE-stained lung tissue of transgenic mice harboring mouse *Egfr*^{de1748-752}. Scale bar = 30 μ m.
- E. CD8-positive lymphocytes were observed in hLR tumors. Scale bar = 30 μ m.
- F. CD8-positive lymphocytes were observed in mDEL tumors. Scale bar = 30 μ m.
- G. Macroscopic and microscopic examination of HE-stained mDEL tumors that were engrafted in the peritoneum in C57BL/6J mice. Scale bar = 30 μ m.
- H. Macroscopic and microscopic examination of HE-stained hLR tumors that were engrafted in the peritoneum in C57BL/6J mice. Scale bar = 30 μ m.
- I. Computed tomography imaging and macroscopic and microscopic examination of HE-stained multiple lung tumors in C57BL/6J mice, in which mDEL tumors had been injected via the tail vein. Scale bar = 30 μ m.

Figure S2

Effects of EGFR-TKIs on tumor growth and metastasis in lung-cancer mouse models.

- A. Effect of afatinib on mDEL tumor growth in syngeneic lung-cancer models; mDEL tumors were treated with 10 mg/kg/day afatinib or vehicle. Error bars, SE.
- B. Efficacy of gefitinib in a lung-metastasis model harboring mouse *Egfr* exon-19 deletion.

Figure S3

Gefitinib-resistant lung tumors harboring secondary *EGFR* mutations.

- A. Direct sequencing of complementary strand for detection of human *EGFR* T790M (c.2369C>T) mutation in gefitinib-resistant hLR tumors.
- B. Expression of MET protein in gefitinib-resistant hLR tumors. GR: gefitinib-resistant hLR tumors.
- C. Phospho-receptor tyrosine kinase array analysis of gefitinib-resistant hLR tumors treated with vehicle, gefitinib 5 mg/kg, gefitinib 50 mg/kg, or osimertinib 5 mg/kg.
- D. Direct sequencing of complementary strand for detection of mouse *Egfr* T792I (c.2375C>T) mutation in gefitinib-resistant mDEL tumors.
- E. Expression of MET protein in gefitinib-resistant mDEL tumors. GR: gefitinib-resistant mDEL tumors.
- F. Phospho-receptor tyrosine kinase array analysis of gefitinib-resistant mDEL tumors treated with vehicle, gefitinib 5 mg/kg, gefitinib 50 mg/kg, or osimertinib 5 mg/kg.

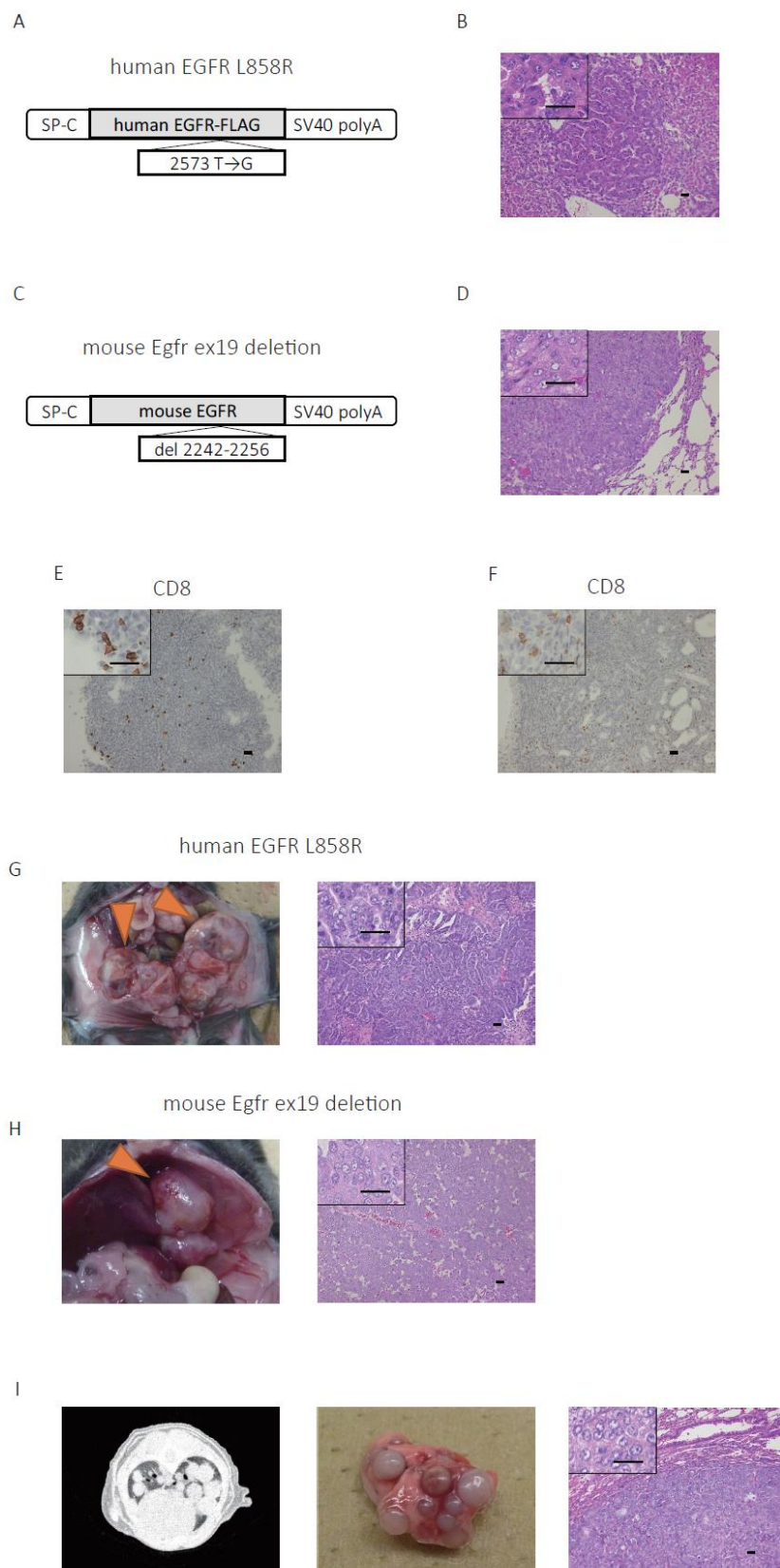
Figure S4

Quantification of EGFR L858R, EGFR T790M and wild type EGFR of cell free DNA using droplet digital PCR. Blue dots indicated EGFR L858R or EGFR T790M mutation. Green dots indicated wild-type EGFR.

Reference

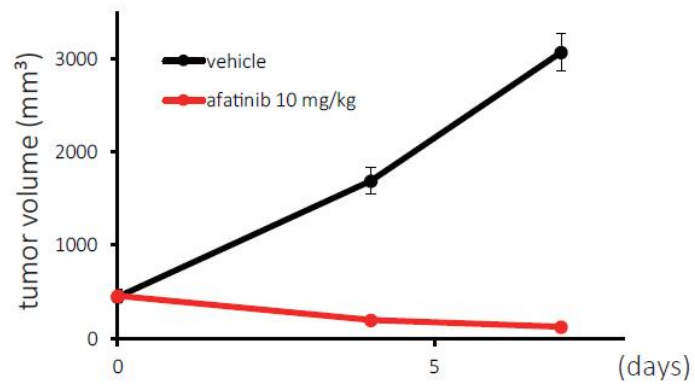
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- [2] Ohashi K, Rai K, Fujiwara Y, et al. Induction of lung adenocarcinoma in transgenic mice expressing activated EGFR driven by the SP-C promoter. *Cancer Sci.* 2008; 99: 1747-53.

Supplementary figure 1

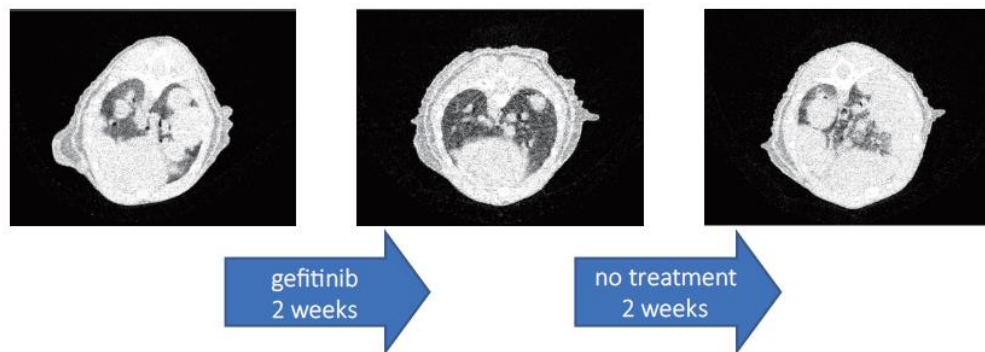


Supplementary figure 2

A

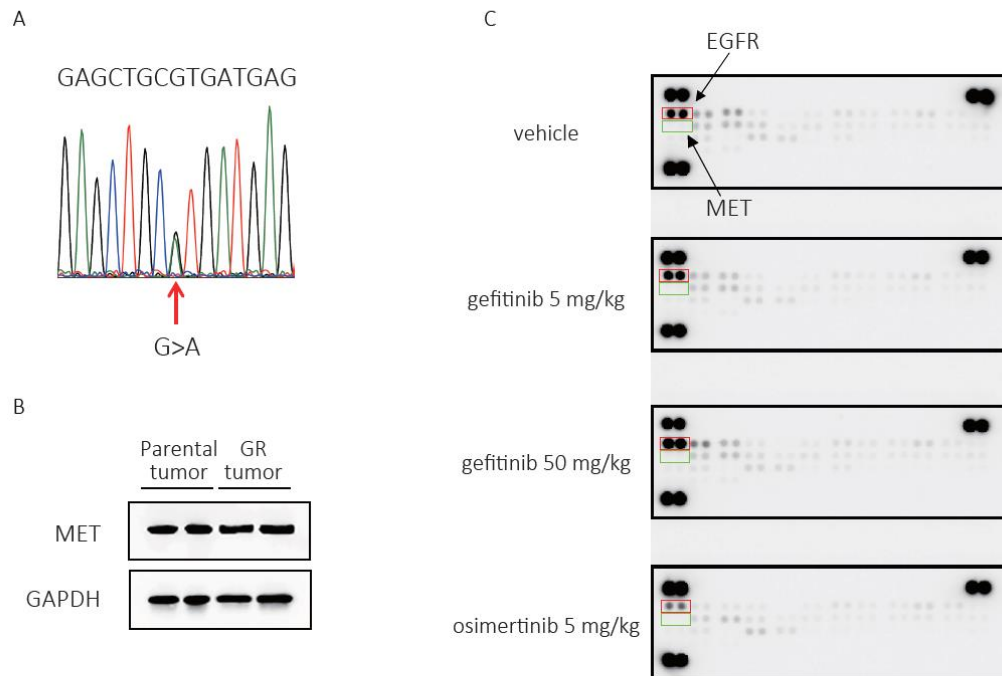


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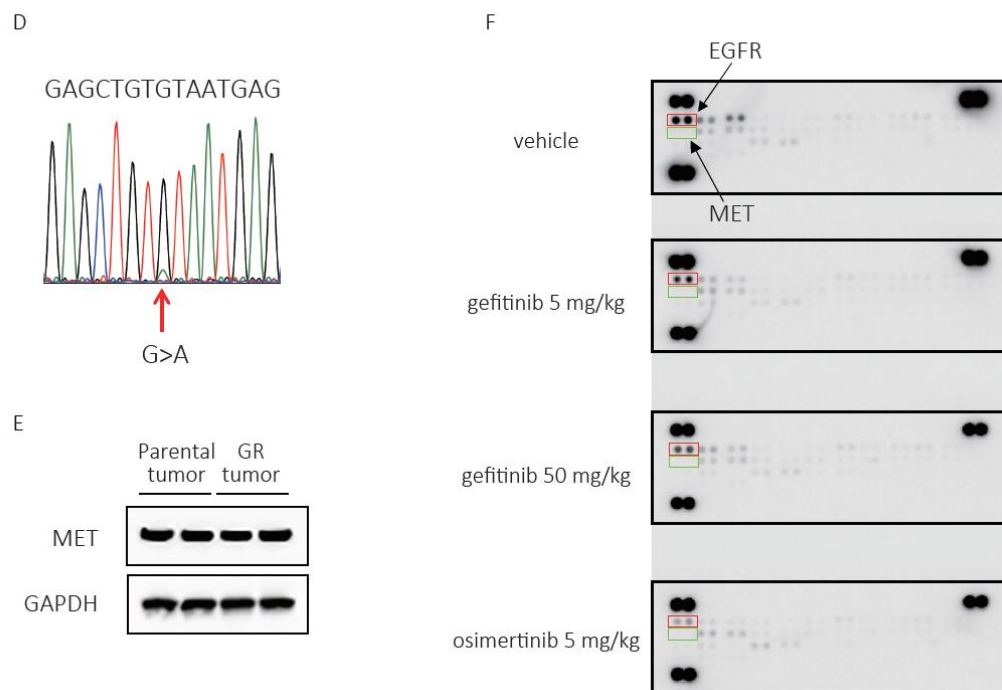


Supplementary figure 3

human EGFR L858R



mouse Egfr ex19 deletion



Supplementary figure 4

