博士学位論文

肺癌細胞株における、EGFR 阻害剤の EMT による耐性機序に及ぼすダサチニブの効果

近畿大学大学院
医学研究科医学系専攻

瀬角裕一

Doctoral Dissertation

Effect of dasatinib on EMT-mediated-mechanism of resistance against EGFR inhibitors in lung cancer cells

Yuichi Sesumi

Department of Surgery, Major in Medical Sciences
Kindai University Graduate School of Medical Sciences
(Director : Prof. Tetsuya Mitsudomi)

November 2017

課博

同　意　書

平成29年10月23日

近畿大学大学院
医学研究科長　殿

共著者 武本智樹 ㊞　　共著者 下治正明 ㊞

共著者 須田健一 ㊞　　共著者 佐藤克明 ㊞

共著者 水内寛 ㊞　　共著者 千葉眞人 ㊞

共著者 小林祥久 ㊞　　共著者 富沢健二 ㊞

共著者 ㊞　　共著者 光冨徹哉 ㊞

論文題目

Effect of dasatinib on EMT-mediated-mechanism of resistance against EGFR inhibitors in lung cancer cells

下記の学位論文提出者が、標記論文を貴学医学博士の学位論文（主論文）として使用することに同意いたします。
また、標記論文を再び学位論文として使用しないことを誓約いたします。

記

1. 学位論文提出者氏名　瀬角裕一
2. 専　攻　分　野　医学系　臓器病態制御外科学Ⅲ

Title: Effect of dasatinib on EMT-mediated-mechanism of resistance against EGFR inhibitors in lung cancer cells

Yuichi Sesumi, Kenichi Suda, Hiroshi Mizuuchi, Yoshihisa Kobayashi, Katsuaki Sato, Masato Chiba, Shimoji Masaki, Kenji Tomizawa, Toshiki Takemoto, Tetsuya Mitsudomi

Division of Thoracic Surgery, Department of Surgery, Kindai University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, 589-8511, Japan.

Running title: Prevention of EMT-Mediated Acquired Resistance in Lung Cancer

Corresponding author: Tetsuya Mitsudomi, MD, Ph.D.
Division of Thoracic Surgery, Department of Surgery, Kindai University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, 589-8511, Japan
Phone: +81-72-366-0221 (ex 3794), FAX: +81-72-367-7771
E-mail: mitsudom@surg.med.kindai.ac.jp

Figures: 3 Figures

Abstract

Objective: The epithelial to mesenchymal transition (EMT) is associated with acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in certain non-small cell lung cancers that harbor *EGFR* mutations. Because no currently available drugs specifically kill cancer cells via EMT, novel treatment strategies that overcome or prevent EMT are needed. A recent report suggested that dasatinib (an ABL/Src kinase inhibitor) inhibits EMT induced by transforming growth factor (TGF)-beta in lung cancer cells (Wilson et al., 2014). In this study, we analyzed effects of dasatinib on the resistance mechanism in HCC4006 cells, which tend to acquire resistance to EGFR-TKIs via EMT.

Materials and methods: Sensitivity to dasatinib in HCC4006 and HCC4006 erlotinib-resistant (ER) cells with an EMT phenotype was analyzed. HCC4006 cells acquired resistance against the combination of erlotinib and dasatinib (HCC4006EDR) following chronic treatment with these drugs. The expression of EMT markers and the resistance mechanism were analyzed.

Results: Short-term or long-term treatment with dasatinib did not reverse EMT in HCC4006ER. In contrast, HCC4006EDR cells maintained an epithelial phenotype, and the mechanism underlying resistance to erlotinib plus dasatinib combination therapy was attributable to a T790M secondary mutation. HCC4006EDR cells, but not HCC4006ER cells, were highly sensitive to a third-generation EGFR-TKI, osimertinib.

Conclusions: Although dasatinib monotherapy did not reverse EMT in HCC4006ER cells, preemptive combination treatment with erlotinib and dasatinib prevented the emergence of acquired resistance via EMT, and led to the emergence of T790M. Our results indicate that preemptive combination therapy may be a promising strategy to

prevent the emergence of EMT-mediated resistance.

Keywords: epithelial-mesenchymal transition, acquired resistance, dasatinib, third-generation EGFR-TKI, non-small cell lung cancer

Introduction

Somatic activating mutations of the epidermal growth factor receptor (*EGFR*) gene are present in approximately 40% and 15% of non-small cell lung cancers (NSCLCs) in East Asians and Caucasians, respectively [1-3]. For patients with NSCLCs harboring an *EGFR* mutation, EGFR-tyrosine kinase inhibitors (TKIs), which are used as a first-line therapy, have demonstrated significantly longer progression-free survival than cytotoxic chemotherapy in randomized phase III studies [4–7]. However, the emergence of acquired resistance is virtually inevitable, even in patients with initially dramatic responses, after a median of approximately one year [8].

Several resistance mechanisms to EGFR-TKIs have been reported, including a secondary point mutation in codon 790 of exon 20 (T790M) of the *EGFR* gene [9], *MET* gene amplification [10,11], *HER2* gene amplification [12,13], transformation to small cell lung cancer [14–17], and epithelial to mesenchymal transition (EMT) [18–21].

Among these resistance mechanisms, T790M is a treatable mutation with the use of the T790M-specific irreversible EGFR-TKIs (third-generation EGFR-TKIs) [22,23]. In contrast, certain resistance mechanisms, particularly EMT, are difficult to treat with currently available agents.

Based on experimental observations, individual lung cancers appear to be “destined” to develop a specific resistance mechanism(s) to EGFR-TKIs [24]. For example, PC9 cells often develop resistance through T790M [25–27], while HCC827 cells acquire *MET* gene amplification to become resistant to the first-generation EGFR-TKIs [10,28–30]. In HCC4006 cells, we and others have identified an EMT

phenotype as the mechanism of acquired resistance to the first-generation EGFR-TKIs [21,31,32]. However, we have previously suggested that this “destiny” can be modified if inhibitors targeted to the “destined” resistance mechanism are given in combination with EGFR-TKIs. For example, HCC827 cells instead acquired resistance through T790M when a MET-TKI was given together with erlotinib [30].

Recently, Wilson et al. reported that dasatinib (an ABL/Src kinase inhibitor) was more effective against lung cancer cells that underwent TGF-beta induced EMT compared to their parent cells [33]. Inspired by this study, we analyzed the effects of dasatinib on HCC4006 erlotinib-resistant (ER) cells as well as HCC4006 cells to determine if dasatinib can prevent EMT as a resistance mechanism.

2. Material and Methods

2.1. Cell lines and reagents

The human lung adenocarcinoma cell line HCC4006 was a kind gift from Dr. A. F. Gazdar (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, Dallas, TX). HCC4006 erlotinib resistant cells (HCC4006ER) were established in our previous study [21]. HCC4006ER cells were chronically treated with dasatinib for 1 month, with medium replacement in every 3 - 4 days. HCC4006 erlotinib and dasatinib resistant cells (HCC4006EDR) were established via chronic exposure to increasing concentrations of erlotinib (20 nM to 2 μM) in the presence of dasatinib as previously described [30]. Cells were

cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) at 37°C in a humidified incubator containing 5% CO_2. Erlotinib (a first-generation reversible EGFR-TKI), dasatinib (an ABL/Src kinase inhibitor) and osimertinib (a third-generation irreversible EGFR-TKI) were purchased from Selleck Chemicals (Houston, TX, U.S.).

2.2. Growth inhibition assay

Cell proliferation was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, cancer cells (4×10^3) were plated onto each well of a 96-well flat-bottomed plate and grown in RPMI containing 10% FBS. After 24 hours, dimethyl sulfoxide (DMSO), erlotinib, dasatinib, osimertinib, or a combination of these drugs was added at the desired drug concentrations, and the cells were incubated for an additional 72 hours. A colorimetric assay was performed after adding 10 μL of Cell Counting Kit-8 reagent to each well and incubating the plates at 37°C for 2–4 hours. The absorbance at 450 nm was read using a multiplate reader (Tecan, Männedorf, Switzerland), and the growth percentage was expressed relative to DMSO-treated controls.

2.3. Preparation of DNA and RNA

Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. Total RNA from cell lines was isolated using a mirVana miRNA Isolation Kit (Qiagen), and complementary DNA was synthesized from total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) according to the manufacturer's protocol.

2.4. Mutation analysis and gene copy number analysis

Mutational analyses of exons 18 to 21 of the *EGFR* gene were conducted by performing direct sequencing. PCR was performed as previously described [21]. Direct sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA,) and an ABI 3130XL instrument (Life Technologies) according to the manufacturer's protocol. The copy numbers of the *MET* and *HER2* genes relative to *LINE1* repetitive elements were measured by real-time PCR using Power SYBR Green PCR Master Mix (Life Technologies) and the StepOnePlus system (Life Technologies). PCR was performed in triplicate for each primer set. Normal genomic DNA was used as a standard sample.

2.5. Western blot analysis

Cells were cultured until subconfluency was reached, and the medium was changed to fresh medium containing DMSO or the indicated drug concentrations. After 8 hours, cell lysates were collected using sodium dodecyl sulfate (SDS) buffer. Approximately 20 μg of total cell lysate was separated by SDS-poly-acrylamide gel

electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). After blocking with Western BLoT Blocking Buffer (Protein Free) (Takara, Shiga, Japan) or PBS containing 2.5% skim milk and 2.5% bovine serum albumin, the membranes were incubated overnight with primary antibodies (1:1,000) and washed with PBS containing 0.05% Tween 20 (PBS-T). Then, the membranes were incubated with a secondary antibody (1:4,000) and washed again with PBS-T before detection with an Amersham ECL Western Blotting Detection Kit (GE Healthcare, Fairfield, CT) or Western BLoT Quant HRP Substrate (Takara). Chemiluminescence was detected with an Amersham Imager 600 instrument (GE Healthcare). Anti-phospho-EGFR, anti-EGFR (Tyr1068), anti-phospho-AKT, anti-AKT(Ser473), anti-phospho-ERK, anti-ERK (Tyr202/Tyr187), anti-E-cadherin, anti-vimentin and anti--actin antibodies were purchased from Cell Signaling Technologies (Danvers, MA).

3. Results

3.1. Dasatinib treatment did not alter the EMT phenotype in HCC4006ER cells

HCC4006ER cells which we had established previously were approximately 1,000-fold more resistant to erlotinib compared with parental HCC4006 cells (Fig. 1A). We first investigated the effects of dasatinib on HCC4006 and HCC4006ER cells. HCC4006ER cells were slightly more sensitive to dasatinib compared with HCC4006 parental cells (IC_{50} values of 0.19 μM and 0.46 μM, respectively) (Fig. 1B). We also investigated the expression of EMT markers by western blot analysis as in the recent studies [34-36]. Neither short-term (8 hours) nor long-term treatment (1 month) with

500nM dasatinib affected E-cadherin and vimentin expression in both parental HCC4006 cells and HCC4006ER cells (Fig. 1C).

3.2. Upfront combination therapy with erlotinib and dasatinib

Next, we examined if upfront combination treatment of dasatinib and erlotinib could prevent the emergence of the EMT phenotype or not. We chronically exposed HCC4006 parental cells to increasing concentrations of erlotinib (20 nM to 2 μM) in the presence of fixed 500 nM dose of dasatinib; the concentration is based on the IC50 of dasatinib (460nM) in HCC4006 cells. Four months later, we were able to obtain resistant cells, which we designated as HCC4006EDR cells (Fig. 2A). HCC4006EDR cell identity was confirmed by a cell-line authentication service using short tandem repeat profiling (Promega, Madison, WI, U.S.).

We analyzed the protein expression of E-cadherin and vimentin in HCC4006, HCC4006ER, and HCC4006EDR cells. HCC4006EDR cells maintained E-cadherin expression and lost vimentin expression, indicating that combination treatment prevented the emergence of EMT-mediated acquired resistance in HCC4006 cells (Fig. 2B).

3.3. Resistance mechanism of HCC4006EDR cells

To analyze the acquired resistance mechanism in HCC4006EDR cells, we investigated currently known resistance mechanisms. HCC4006EDR cells did not

exhibit *MET* gene amplification or *HER2* gene amplification (Fig. 2C). However, we observed a T790M secondary mutation in HCC4006EDR cells (Fig. 2D). As expected, HCC4006EDR cells were highly sensitive to osimertinib (Fig. 3A), and EGFR downstream pathways were notably inhibited by osimertinib treatment in HCC4006EDR cells (Fig. 3B).

4. Discussion

We demonstrated that upfront combination therapy with dasatinib prevented the emergence of EMT-mediated acquired resistance in HCC4006 cells. Cells resistant to combination therapy acquired the T790M secondary mutation, which is highly sensitive to osimertinib.

EMT is a biological process through which epithelial cells lose their polarity and cell-cell adhesions and acquire mesenchymal phenotypes characterized by higher motility, invasion, and metastases [37–39]. EMT is also the reported cause of resistance to therapies such as molecular targeted drugs [20,21], cytotoxic agents [40–43], and radiation [44]. Given that no currently available drugs specifically kill cancer cells with EMT, the identification of novel treatment strategies that prevent EMT would be promising.

Similar attempts have recently been reported. Soucheray et al. utilized a TGF-beta inhibitor (SB431542) in combination with erlotinib in HCC4006 cells and found that resistant cells acquired the T790M secondary mutation [35]. This is consistent with the fact that TGF-beta regulated genes were upregulated in HCC4006ER cells compared with the parental cells by the gene set enrichment analysis [21].

The aforementioned results by Soucheray, et al., together with ours, suggest that EMT inhibitors are able to alter the resistance mechanism from EMT to the T790M secondary mutation. With the availability of third-generation EGFR-TKIs, T790M-mediated resistance became far easier to deal with. Therefore, upfront combination therapy preventing EMT and instead guiding cancer cells to the T790M secondary mutation would result in prolonged overall survival. This phenomenon is similar to what we reported previously: the upfront combination of PHA-665752 (MET-TKI) plus erlotinib resulted in T790M in HCC827 cells, which otherwise become resistant via *MET* amplification without PHA-665752 [30].

When considering combination therapy, toxicities is a concern. Currently, there are no available data regarding the combination of TGF-beta receptor inhibitors and EGFR-TKIs. However, the combination therapy of erlotinib with dasatinib was conducted in a phase Ⅰ/II clinical trial [45]. Although this trial did not focus on lung cancer patients with *EGFR* mutations, the results showed tolerable adverse events by this combination.

The frequency of the resistance through EMT has been incompletely clarified. Sequist et al. reported that EMT occurred in 3 patients out of seven without known other resistance mechanisms, and none out of five patients with T790M mutation after acquisition of resistance to first-generation EGFR-TKIs [14]. While, Uramoto et al. reported that EMT was observed in 4/9 cases of resistance to EGFR-TKI, independent of T790M mutation [18]. At this time, it is not possible to predict which patients become resistant through EMT. In the future, however, we may be able to predict such patients and whether they would be candidates for dasatinib and erlotinib combination therapy. Thus far, it is unclear if resistant cells with EMT are

derived from the “selection” of pre-existing minor EMT clones or from the “acquisition” of the EMT phenotype by epithelial cells that persist during early EGFR-TKI treatment. However, it can be speculated that the clinical application of third-generation EGFR-TKIs that inhibit the emergence the T790M mutation results in a higher incidence of acquired resistance via EMT.

In conclusion, targeting the resistance mechanism after the acquisition of resistance to EGFR-TKIs is one important strategy to improve outcomes for patients with *EGFR* mutations. However, for those resistance mechanisms for which there is no specific treatment, including EMT, preemptive combination therapy should be a promising strategy to guide cancer cells towards treatable resistance.

Conflict of interest

Tetsuya Mitsudomi received lecture fee from AstraZeneca, Boehringer-Ingelheim, Chugai and Pfizer and research funding from AstraZeneca, Boehringer-Ingelheim and Chugai. All other authors declare that they have no conflict of interest related to this study.

Fundings

This work was partially supported by grants from the IASLC Lung Cancer Young Investigator Award for 2015 - 2017 (K.S.) and the Grants-in-Aid of Cancer Research from the Ministry of Education, Science, Sports, and Culture of Japan (15K18450 to K.S.).

Acknowledgments

The authors are grateful to Dr. Takeshi Yoshida (Department of Medical Oncology, Kindai University Faculty of Medicine) for helpful discussions regarding this article. The authors also thank Mr. Yoshihiro Mine, Ms. Eiko Honda (Center for instrumental Analyses Central Research Facilities, Kindai University Faculty of Medicine) for their technical assistance.

Reference

[1] Y. Yatabe, K.M. Kerr, A. Utomo, P. Rajadurai, V. Tran, X. Du, et al., EGFR mutation testing practices within the Asia Pacific region: results of a multicenter diagnostic survey, Journal of Thoracic Oncology. 10 (2015) 438–445.

[2] M. Kris, B. Johnson, L. Berry, D. Kwiatkowski, A. Iafrate, I. Wistuba, et al., Using Multiplexed Assays of Oncogenic Drivers in Lung Cancers to Select Targeted Drugs, Jama. 311 (2014) 1998–2006.

[3] K. Suda, K. Tomizawa, T. Mitsudomi, Biological and clinical significance of KRAS mutations in lung cancer: an oncogenic driver that contrasts with EGFR mutation., Cancer Metastasis Rev. 29 (2010) 49–60.

[4] M. Maemondo, A. Inoue, K. Kobayashi, S. Sugawara, S. Oizumi, H. Isobe, et al., Gefitinib or Chemotherapy for Non–Small-Cell Lung Cancer with Mutated EGFR, New Engl J Medicine. 362 (2010) 2380–2388.

[5] T. Mitsudomi, S. Morita, Y. Yatabe, S. Negoro, I. Okamoto, J. Tsurutani, et al., Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial, Lancet Oncol. 11 (2010) 121–128.

[6] C. Zhou, Y.-L. Wu, G. Chen, J. Feng, X.-Q. Liu, C. Wang, et al., Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study, Lancet Oncol. 12 (2011) 735–742.

[7] R. Rosell, E. Carcereny, R. Gervais, A. Vergnenegre, B. Massuti, E. Felip, et al., Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial, Lancet Oncol. 13 (2012) 239–246.

[8] S. Morita, T. Hirashima, K. Hagiwara, T. Hida, N. Sunaga, K. Sugio, et al., Gefitinib combined survival analysis of the mutation positives from the prospective phase II trials (I-CAMP), 26 (2008) 8101.

[9] D.R. Camidge, W. Pao, L.V. Sequist, Acquired resistance to TKIs in solid tumours: learning from lung cancer., Nat Rev Clin Oncol. 11 (2014) 473–81.

[10] J.A. Engelman, K. Zejnullahu, T. Mitsudomi, Y. Song, C. Hyland, J.O. Park, et al., MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling., Science. 316 (2007) 1039–43.

[11] J. Bean, C. Brennan, J.-Y.Y. Shih, G. Riely, A. Viale, L. Wang, et al., 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. 104 (2007)

20932–7.
[12] K. Takezawa, V. Pirazzoli, M.E. Arcila, C.A. Nebhan, X. Song, E. de Stanchina, et al., HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR-mutant lung cancers that lack the second-site EGFRT790M mutation., Cancer Discov. 2 (2012) 922–33.
[13] H. Yu, M. Arcila, N. Rekhtman, C. Sima, M. Zakowski, W. Pao, et al., Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers., Clinical Cancer Research : An Official Journal of the American Association for Cancer Research. 19 (2013) 2240–7.
[14] L. Sequist, B. Waltman, D. Dias-Santagata, S. Digumarthy, A. Turke, P. Fidias, et al., Genotypic and Histological Evolution of Lung Cancers Acquiring Resistance to EGFR Inhibitors, Science Translational Medicine. 3 (2011) 75ra26–75ra26.
[15] V. Fallet, A.-M. Ruppert, V. Poulot, R. Lacave, L. Belmont, M. Antoine, et al., Secondary Resistance to Erlotinib: Acquired T790M Mutation and Small-Cell Lung Cancer Transformation in the Same Patient, J Thorac Oncol. 7 (2012) 1061–1063.
[16] S. Watanabe, T. Sone, T. Matsui, K. Yamamura, M. Tani, A. Okazaki, et al., Transformation to small-cell lung cancer following treatment with EGFR tyrosine kinase inhibitors in a patient with lung adenocarcinoma., Lung Cancer. 82 (2013) 370–2.
[17] K. Suda, I. Murakami, K. Sakai, H. Mizuuchi, S. Shimizu, K. Sato, et al., Small cell lung cancer transformation and T790M mutation: complimentary roles in acquired resistance to kinase inhibitors in lung cancer., Sci Rep. 5 (2015) 14447.
[18] H. Uramoto, T. Iwata, T. Onitsuka, H. Shimokawa, T. Hanagiri, T. Oyama, Epithelial-mesenchymal transition in EGFR-TKI acquired resistant lung adenocarcinoma., Anticancer Res. 30 (2010) 2513–7.
[19] J.-H. Chung, J. Rho, X. Xu, J. Lee, H. Yoon, C. Lee, et al., Clinical and molecular evidences of epithelial to mesenchymal transition in acquired resistance to EGFR-TKIs., Lung Cancer (Amsterdam, Netherlands). 73 (2011) 176–82.
[20] J. Rho, Y. Choi, J. Lee, B.-Y. Ryoo, I. Na, S. Yang, et al., Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line, Lung Cancer. 63 (2009) 219–226.
[21] K. Suda, K. Tomizawa, M. Fujii, H. Murakami, H. Osada, Y. Maehara, et al., Epithelial to mesenchymal transition in an epidermal growth factor receptor-mutant lung cancer cell line with acquired resistance to erlotinib., J Thorac Oncol. 6 (2011) 1152–61.
[22] P.A. Jänne, J.C. Yang, D.-W.W. Kim, D. Planchard, Y. Ohe, S.S. Ramalingam, et al., AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer., N. Engl. J. Med. 372

(2015) 1689–99.
[23] L.V. Sequist, L. Rolfe, A.R. Allen, Rociletinib in EGFR-Mutated Non-Small-Cell Lung Cancer., N. Engl. J. Med. 373 (2015) 578–9.
[24] K. Suda, H. Mizuuchi, Y. Maehara, T. Mitsudomi, Acquired resistance mechanisms to tyrosine kinase inhibitors in lung cancer with activating epidermal growth factor receptor mutation--diversity, ductility, and destiny., Cancer Metastasis Rev. 31 (2012) 807–14.
[25] A. Ogino, H. Kitao, S. Hirano, A. Uchida, M. Ishiai, T. Kozuki, et al., Emergence of epidermal growth factor receptor T790M mutation during chronic exposure to gefitinib in a non small cell lung cancer cell line., Cancer Res. 67 (2007) 7807–14.
[26] D. Ercan, K. Zejnullahu, K. Yonesaka, Y. Xiao, M. Capelletti, A. Rogers, et al., Amplification of EGFR T790M causes resistance to an irreversible EGFR inhibitor., Oncogene. 29 (2010) 2346–56.
[27] T. Yoshida, I. Okamoto, W. Okamoto, E. Hatashita, Y. Yamada, K. Kuwata, et al., Effects of Src inhibitors on cell growth and epidermal growth factor receptor and MET signaling in gefitinib-resistant non-small cell lung cancer cells with acquired MET amplification., Cancer Sci. 101 (2010) 167–72.
[28] T.G. Bivona, H. Hieronymus, J. Parker, K. Chang, M. Taron, R. Rosell, et al., FAS and NF-κB signalling modulate dependence of lung cancers on mutant EGFR., Nature. 471 (2011) 523–6.
[29] A.B. Turke, K. Zejnullahu, Y.-L.L. Wu, Y. Song, D. Dias-Santagata, E. Lifshits, et al., Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC., Cancer Cell. 17 (2010) 77–88.
[30] K. Suda, I. Murakami, T. Katayama, K. Tomizawa, H. Osada, Y. Sekido, et al., Reciprocal and complementary role of MET amplification and EGFR T790M mutation in acquired resistance to kinase inhibitors in lung cancer., Clinical Cancer Research : An Official Journal of the American Association for Cancer Research. 16 (2010) 5489–98.
[31] K. Ohashi, L. Sequist, M. Arcila, T. Moran, J. Chmielecki, Y.-L. Lin, et al., Lung cancers with acquired resistance to EGFR inhibitors occasionally harbor BRAF gene mutations but lack mutations in KRAS, NRAS, or MEK1., Proceedings of the National Academy of Sciences of the United States of America. 109 (2012) E2127–33.
[32] K. Shien, S. Toyooka, H. Yamamoto, J. Soh, M. Jida, K.L. Thu, et al., Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells., Cancer Res. 73 (2013) 3051–61.
[33] C. Wilson, K. Nicholes, D. Bustos, E. Lin, Q. Song, J.-P.P. Stephan, et al., Overcoming EMT-associated resistance to anti-cancer drugs via Src/FAK pathway

418 inhibition., Oncotarget. 5 (2014) 7328–41.
419 [34] K. Kitamura, M. Seike, T. Okano, K. Matsuda, A. Miyanaga, H. Mizutani, et al.,
420 MiR-134/487b/655 cluster regulates TGF-β-induced epithelial-mesenchymal transition and
421 drug resistance to gefitinib by targeting MAGI2 in lung adenocarcinoma cells., Mol. Cancer
422 Ther. 13 (2014) 444–53.
423 [35] M. Soucheray, M. Capelletti, I. Pulido, Y. Kuang, C.P. Paweletz, J.H. Becker, et
424 al., Intratumoral Heterogeneity in EGFR-Mutant NSCLC Results in Divergent Resistance
425 Mechanisms in Response to EGFR Tyrosine Kinase Inhibition., Cancer Res. 75 (2015)
426 4372–83.
427 [36] C. Hwangbo, N. Tae, S. Lee, O. Kim, O.K. Park, J. Kim, et al., Syntenin regulates
428 TGF-β1-induced Smad activation and the epithelial-to-mesenchymal transition by inhibiting
429 caveolin-mediated TGF-β type I receptor internalization., Oncogene. 35 (2016) 389–401.
430 [37] J.P. Thiery, Epithelial-mesenchymal transitions in development and pathologies.,
431 Curr. Opin. Cell Biol. 15 (2003) 740–6.
432 [38] M.J. Wheelock, K.R. Johnson, Cadherins as modulators of cellular phenotype.,
433 Annu. Rev. Cell Dev. Biol. 19 (2003) 207–35.
434 [39] C.L. Chaffer, R.A. Weinberg, A perspective on cancer cell metastasis., Science.
435 331 (2011) 1559–64.
436 [40] W.-L.L. Zhuo, Y. Wang, X.-L.L. Zhuo, Y.-S.S. Zhang, Z.-T.T. Chen, Short
437 interfering RNA directed against TWIST, a novel zinc finger transcription factor, increases
438 A549 cell sensitivity to cisplatin via MAPK/mitochondrial pathway., Biochem. Biophys. Res.
439 Commun. 369 (2008) 1098–102.
440 [41] W. Zhuo, Y. Wang, X. Zhuo, Y. Zhang, X. Ao, Z. Chen, Knockdown of Snail, a
441 novel zinc finger transcription factor, via RNA interference increases A549 cell sensitivity to
442 cisplatin via JNK/mitochondrial pathway., Lung Cancer. 62 (2008) 8–14.
443 [42] S. Thomson, E. Buck, F. Petti, G. Griffin, E. Brown, N. Ramnarine, et al., Epithelial
444 to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma
445 cell lines and xenografts to epidermal growth factor receptor inhibition., Cancer Res. 65
446 (2005) 9455–62.
447 [43] H. Mizuuchi, K. Suda, K. Sato, S. Tomida, Y. Fujita, Y. Kobayashi, et al., Collateral
448 chemoresistance to anti-microtubule agents in a lung cancer cell line with acquired
449 resistance to erlotinib., PLoS ONE. 10 (2015) e0123901.
450 [44] Y. Shintani, A. Okimura, K. Sato, T. Nakagiri, Y. Kadota, M. Inoue, et al., Epithelial
451 to mesenchymal transition is a determinant of sensitivity to chemoradiotherapy in non-small
452 cell lung cancer., The Annals of Thoracic Surgery. 92 (2011) 1794–804; discussion 1804.
453 [45] E.B. Haura, T. Tanvetyanon, A. Chiappori, C. Williams, G. Simon, S. Antonia, et al.,

Phase I/II study of the Src inhibitor dasatinib in combination with erlotinib in advanced non-small-cell lung cancer., J. Clin. Oncol. 28 (2010) 1387–1394.

Figure Legends

Figure 1. Effect of dasatinib treatment on HCC4006 and HCC4006ER cells.

(A and B) Anti-proliferative effects of erlotinib (A) and dasatinib (B) in HCC4006 and HCC4006ER cells. Four thousand cells were incubated for 24 hours and treated with the indicated concentrations of each drug for additional 72 hours.

(C) Western blot analysis of E-cadherin and Vimentin in HCC4006 and HCC4006ER cells. Both cells were treated with 500nM dasatinib for 8 hours or 1 month.

Figure 2. Analysis of the resistance mechanism of HCC4006EDR cells.

(A) Anti-proliferative effects of the combination of erlotinib and dasatinib in HCC4006, HCC4006ER and HCC4006EDR cells.

Four thousand cells were incubated for 24 hours and treated with the combination of 0.5μM dasatinib and indicated concentrations of erlotinib for additional 72 hours.

(B) Western blot analysis of E-cadherin and vimentin in each cell line. In HCC4006EDR cells, E-cadherin expression was increased and vimentin expression was decreased compared with HCC4006ER cells.

(C) Gene copy numbers of *MET* and *HER2* in HCC4006EDR cells. Data are expressed relative to *LINE-1* elements. *MET* and *HER2* gene copy numbers of each cell line were measured by quantitative real-time PCR.

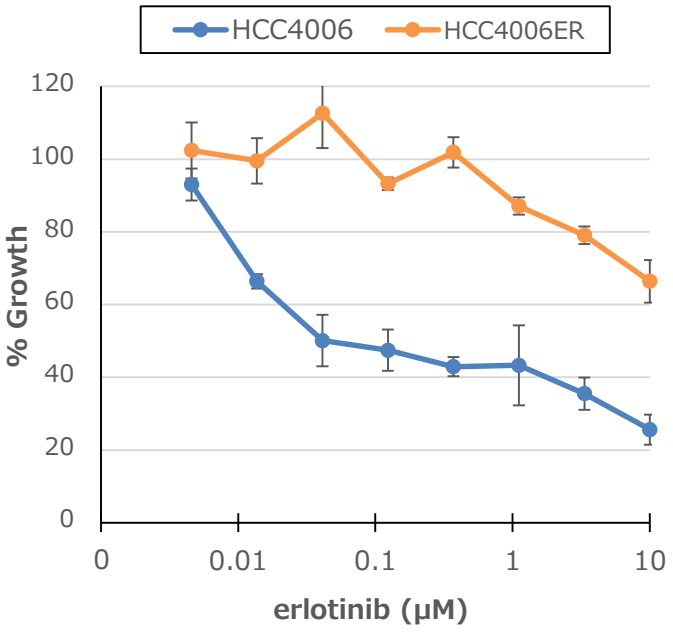
(D) Antisense strands of sequencing chromatograms for *EGFR* exon 20 are shown. C to T substitution at nucleotide 2369 (G to A on the antisense strand) resulted in the T790M mutation.

Figure 3. In vitro sensitivity to the third-generation EGFR-TKI osimertinib in HCC4006EDR cells.

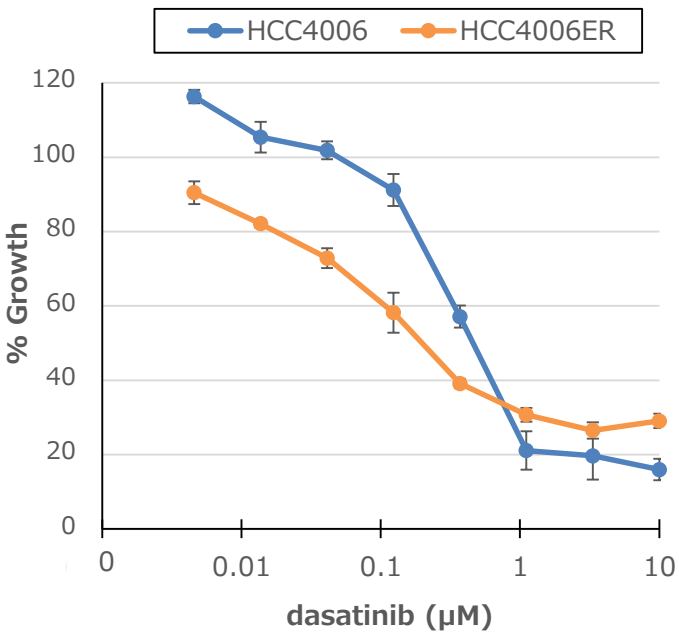
(A) Anti-proliferative effects of osimertinib in HCC4006, HCC4006ER and HCC4006EDR cells. Four thousand cells were incubated for 24 hours and treated with the indicated concentrations of osimertinib for additional 72 hours.

(B) Western blot analysis of EGFR and its downstream signaling components in HCC4006, HCC4006ER and HCC4006EDR cells. Total cell lysates were extracted 8 hours after exposure to DMSO (D), erlotinib (E; 2 μM) or osimertinib (O; 160 nM).

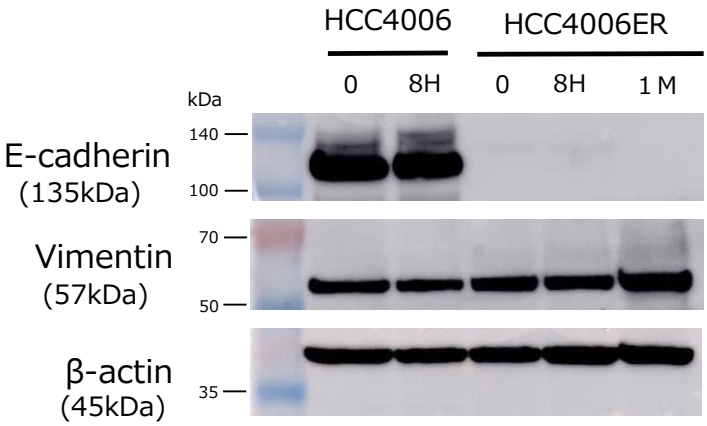
A

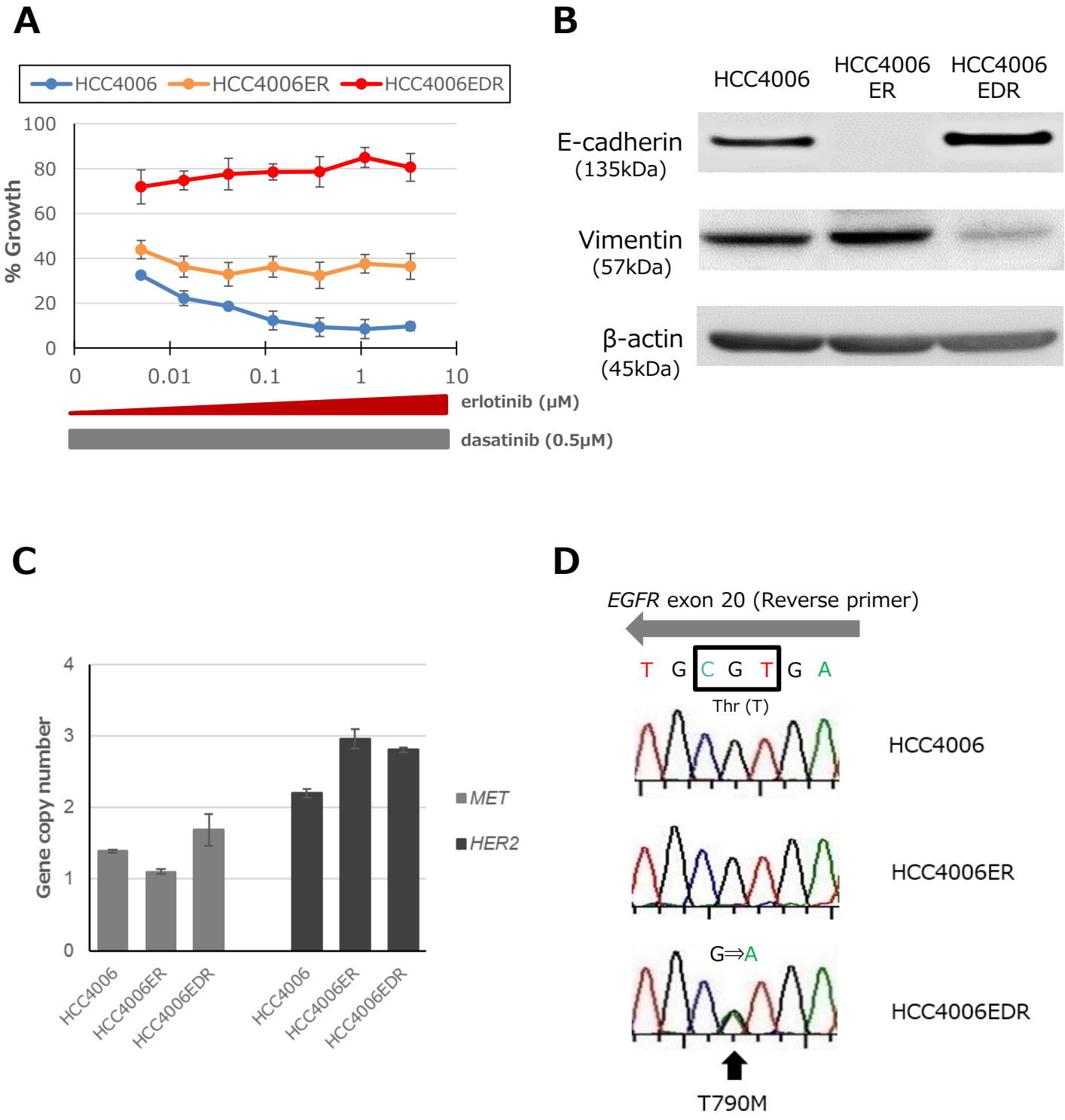

HCC4006
HCC4006ER
120
100
80
60
40
20
0
% Growth
0
0.01
0.1
1
10
erlotinib (μM)

B

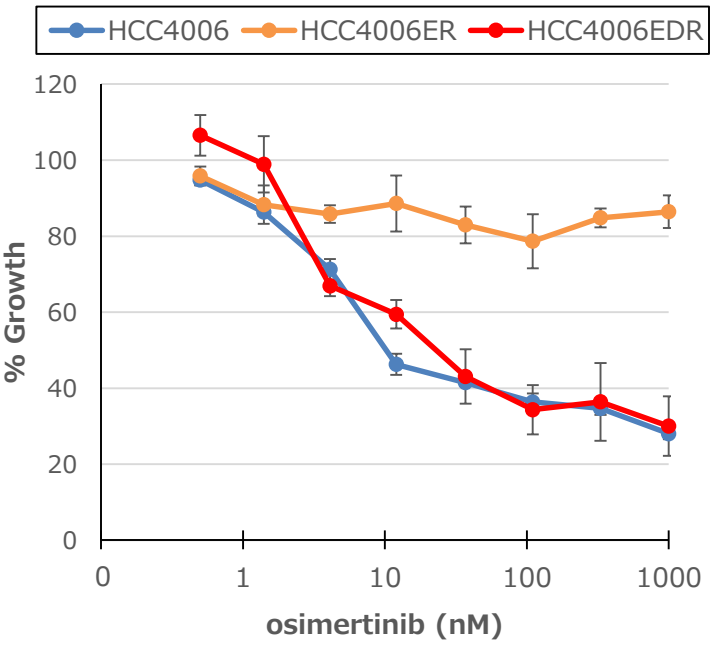

HCC4006
HCC4006ER
120
100
80
60
40
20
0
% Growth
0
0.01
0.1
1
10
dasatinib (μM)

C

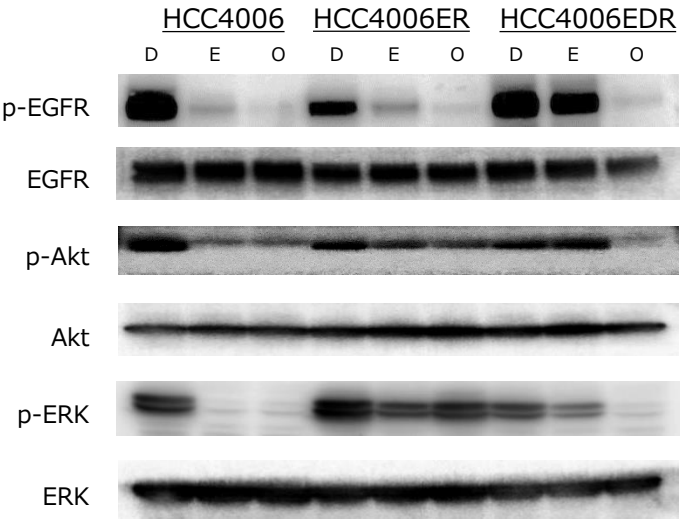

HCC4006
HCC4006ER
0
8H
0
8H
1 M
kDa
140
100
E-cadherin
(135kDa)
70
50
Vimentin
(57kDa)
35
β-actin
(45kDa)


A
HCC4006
HCC4006ER
HCC4006EDR
% Growth
100
80
60
40
20
0
0
0.01
0.1
1
10
erlotinib (μM)
dasatinib (0.5μM)
B
HCC4006
HCC4006 ER
HCC4006 EDR
E-cadherin
(135kDa)
Vimentin
(57kDa)
β-actin
(45kDa)
C
Gene copy number
4
3
2
1
0
MET
HER2
HCC4006
HCC4006ER
HCC4006EDR
HCC4006
HCC4006ER
HCC4006EDR
D
EGFR exon 20 (Reverse primer)
T G C G T G A
Thr (T)
HCC4006
HCC4006ER
G⇒A
HCC4006EDR
T790M

A


HCC4006
HCC4006ER
HCC4006EDR
120
100
80
60
40
20
0
% Growth
0
1
10
100
1000
osimertinib (nM)

B


HCC4006
HCC4006ER
HCC4006EDR
D
E
O
D
E
O
D
E
O
p-EGFR
EGFR
p-Akt
Akt
p-ERK
ERK