Lung Cancer 127 (2019) 146–152

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Efficacy of afatinib or osimertinib plus cetuximab combination therapy for non-small-cell lung cancer with *EGFR* exon 20 insertion mutations

Hanako Hasegawa[a], Hiroyuki Yasuda[a,*], Junko Hamamoto[a], Keita Masuzawa[a], Tetsuo Tani[a], Shigenari Nukaga[a], Toshiyuki Hirano[a], Keigo Kobayashi[a], Tadashi Manabe[a], Hideki Terai[b], Shinnosuke Ikemura[a,c], Ichiro Kawada[a], Katsuhiko Naoki[c], Kenzo Soejima[a]

[a] Division of Pulmonary Medicine, Department of Medicine, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan
[b] Kitasato Institute Hospital, Department of Internal Medicine, 5-9-1 Shirokane, Minatoku, Tokyo, 108-8642, Japan
[c] Keio Cancer Center, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan

ARTICLE INFO

Keywords:
EGFR tyrosine kinase inhibitor
EGFR exon 20 insertion mutations
Lung cancer
Afatinib
Cetuximab

ABSTRACT

Objectives: Epidermal growth factor receptor (EGFR) mutation-positive lung cancer accounts for a significant subgroup of non-small cell lung cancers (NSCLC). Approximately 4–10% of *EGFR* mutations in NSCLC are *EGFR* exon 20 insertion mutations, which are reportedly associated with resistance to EGFR tyrosine kinase inhibitor (EGFR-TKI) treatment. NSCLC patients carrying these mutations are rarely treated with EGFR-TKIs. The purpose of this study was to evaluate the efficacy of afatinib or osimertinib plus cetuximab combination therapy in experimental NSCLC models with *EGFR* exon 20 insertion mutations.

Materials and methods: The *EGFR* mutations examined in this study were A763_Y764insFQEA, Y764_V765insHH, A767_V769dupASV, and D770_N771insNPG. Ba/F3 cells constitutively expressing wild type or mutated *EGFR* were used to determine the efficacy of afatinib or osimertinib plus cetuximab combination therapy *in vitro*. To determine the efficacy of the combination therapy *in vivo,* female BALB/c-nu mice were injected subcutaneously with 1 million Ba/F3 cells carrying EGFR A767_V769dupASV or Y764_V765insHH.

Results: We observed a mild but significant ($P < 0.05$) additive effect of the combination therapy against several *EGFR* exon 20 insertion mutations *in vitro*. Regarding *EGFR* A767_V769dupASV and *EGFR* Y764_V765insHH, cetuximab and afatinib single treatment did not induce significant inhibition of tumor formation; however, afatinib plus cetuximab combination treatment induced significant ($P < 0.05$) tumor growth inhibition without significant body weight loss or skin rash.

Conclusion: The combination therapy induced a more potent inhibitory effect against several *EGFR* exon 20 insertion mutations than either therapy alone. Cetuximab can potentially increase the efficacy of afatinib or osimertinib in NSCLC with *EGFR* exon 20 insertion mutations.

1. Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide [1]. However, in recent decades, the elucidation of key molecular mechanisms contributed to the improvement of prognosis in patients with lung cancer, especially in those with non-small cell lung cancer (NSCLC) [2]. In 2004, an association between somatic mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) gene and the response to EGFR tyrosine kinase inhibitor (TKI) treatment were reported by several groups [3,4]. Among the *EGFR* mutations found in NSCLC, in-frame deletions near the LREA motif in exon 19 (exon 19 deletions) and the L858R point mutation in exon 21 are classic mutations, accounting for approximately 80–90% of *EGFR* mutations [5]. These mutations destabilize the inactive conformation of EGFR and induce its constitutive activation [6–8]. Activated EGFR transduces downstream signaling, including the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/protein kinase B (AKT) pathways [5,9]. After this discovery, multiple clinical trials demonstrated the efficacy of EGFR-TKIs in NSCLC patients carrying classic *EGFR* mutations [10–13]. Multiple EGFR-TKIs, including gefitinib, erlotinib (first-generation), afatinib, dacomitinib (second-generation), and osimertinib (third-

* Corresponding author at: Division of Pulmonary Medicine, Department of Medicine, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan.
E-mail address: hiroyukiyasuda@a8.keio.jp (H. Yasuda).

https://doi.org/10.1016/j.lungcan.2018.11.039
Received 13 October 2018; Received in revised form 24 November 2018; Accepted 28 November 2018

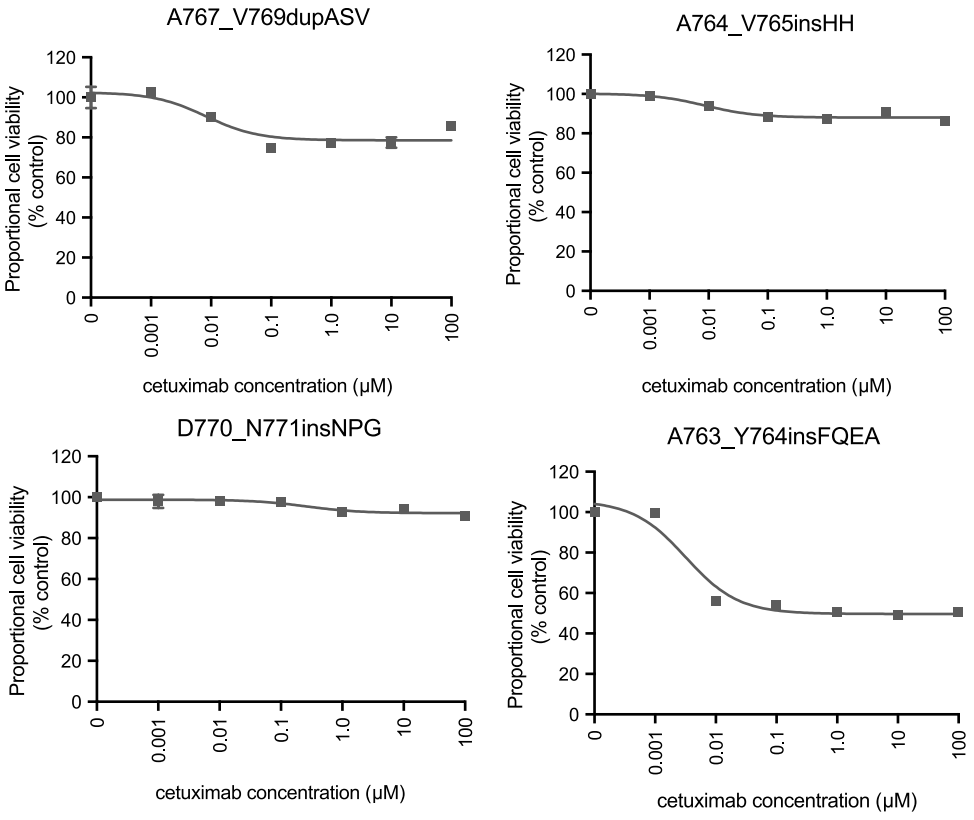

Fig. 1. Effect of cetuximab on Ba/F3 cells harboring *EGFR* exon 20 insertion mutations. The sensitivity of Ba/F3 cells expressing *EGFR* exon 20 insertion mutations to cetuximab was examined. MTS assays were conducted with Ba/F3 cells expressing the indicated *EGFR* genotypes. Data points represent the mean ± standard deviation.

generation), are in clinical use for the treatment of NSCLC patients with these *EGFR* mutations [14–16]. However, the *EGFR* exon 20 insertion mutations are reportedly associated with resistance to EGFR-TKIs [17,18]. One exception is A763_Y764insFQEA, which we identified as an EGFR-TKI sensitive *EGFR* exon 20 insertion mutation [18]. *EGFR* exon 20 insertion mutations account for approximately 4–10% of all *EGFR* mutations associated with NSCLC [19]. Afatinib had a response rate of 8.3% in *EGFR* exon 20 insertion mutation-positive NSCLC, indicating the limited efficacy of EGFR-TKIs against this subgroup of NSCLC [19]. To improve the prognosis in NSCLC patients harboring *EGFR* exon 20 insertion mutations, the development of an effective therapy is urgently needed. Our study findings show that cetuximab combination therapy would be a potential strategy for the treatment of these patients.

2. Materials and methods

2.1. Reagents

Erlotinib and afatinib were purchased from LC Laboratories (Woburn, MA, USA). Osimertinib was purchased from Selleck Chemicals (Houston, TX, USA). Cetuximab was purchased from Keio University Hospital (Tokyo, Japan). Total EGFR antibody (#2232), total AKT antibody (#9272), phospho-AKT (S473; D9E) antibody (#4060), total p44/42 MAPK antibody (#9102S), and phospho-p44/42 MAPK (T202/204) antibody (#9101S) were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-EGFR (Y1068) antibody (44,788 G) was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Ba/F3 stable cell lines

Ba/F3 cells constitutively expressing wild type or mutated *EGFR* were created as previously described [18]. Ba/F3 cells harboring *EGFR* mutations were cultured in RPMI-1640 growth medium supplemented with 10% fetal bovine serum, at 37 °C in a humidified 5% CO_2 incubator. Ba/F3 cells expressing *EGFR* wild type were cultured in RPMI-1640 growth medium supplemented with 10 ng/mL epidermal growth factor (EGF) and 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. The *EGFR* mutations examined in this study were A763_Y764insFQEA, Y764_V765insHH, A767_V769dupASV, and D770_N771insNPG.

2.3. Cell proliferation assay

The MTS assay was performed as previously described [18]. Ba/F3 cells were seeded with or without EGFR-TKI in 96-well plates. Control cells were treated with the same concentration of vehicle, dimethyl sulfoxide (DMSO). Absorbance was measured after 72 h of treatment period. All experiments were performed at least three times.

2.4. Immunoblotting analysis by western blotting

The cells were treated with 0.1 μmol/L of EGFR-TKI. Cetuximab was used at a concentration of 10 μg/mL. The cells were subjected to single or combination therapy for 8 and 24 h, followed by lysis in Cell Lysis Buffer (Cell Signaling Technology). Equal amounts of protein were loaded per lane on sodium dodecyl sulfate-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated overnight with primary antibodies at 4 °C and then incubated with secondary antibodies for 1 h. For the detection of proteins, the membranes were incubated with agitation in LumiGLO reagent and peroxide (Cell Signaling Technology) and then exposed to X-ray film.

2.5. Apoptosis assay

Ba/F3 cells carrying *EGFR* A763_Y764insFQEA, Y764_V765insHH, A767_V769dupASV, and D770_N771insNPG were seeded in 6-well

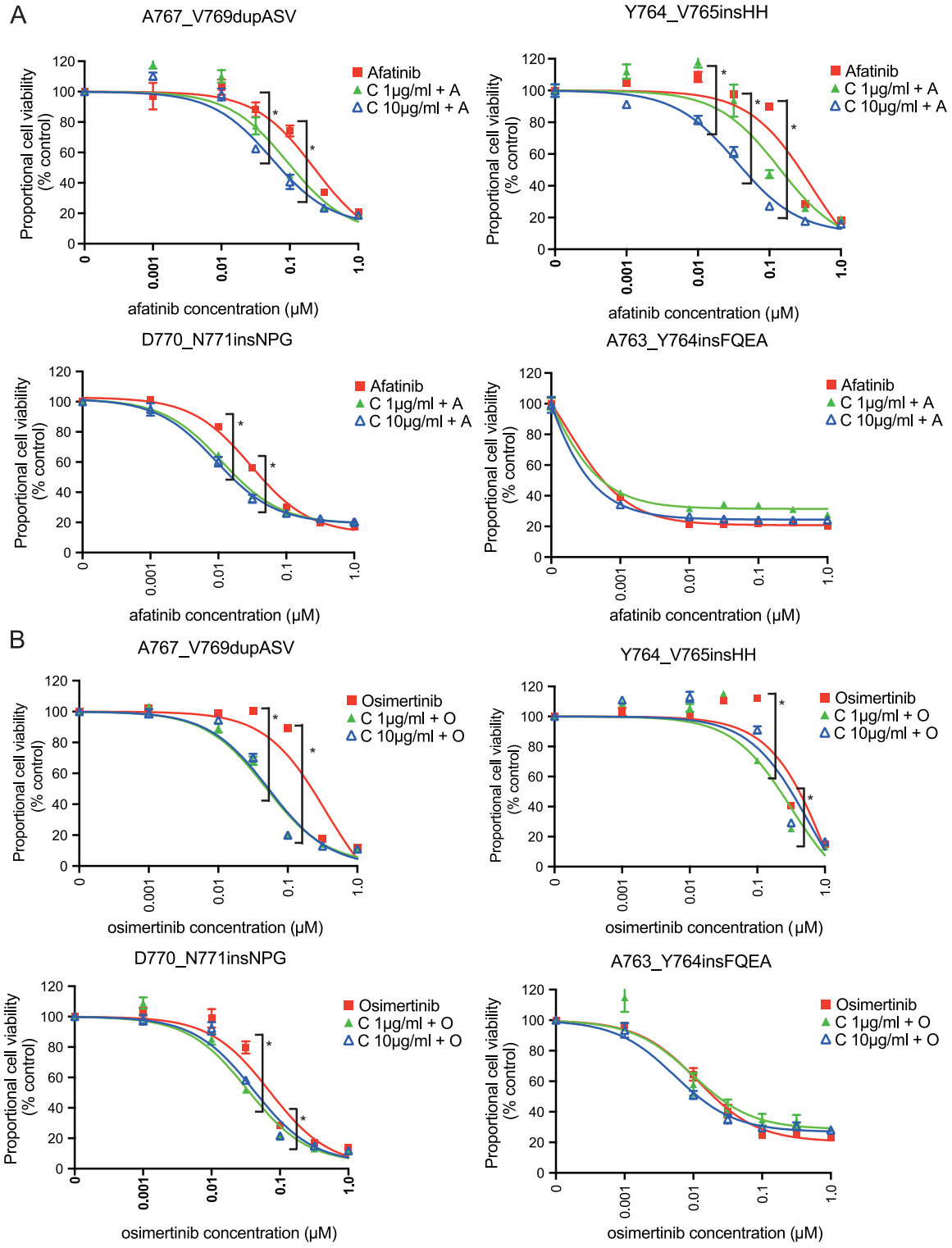

Fig. 2. Additional efficacy of afatinib and cetuximab combination therapy.
MTS assays were conducted with Ba/F3 cells expressing the indicated *EGFR* exon 20 insertion mutations. (A) Cells were treated with afatinib (A) and/or cetuximab (C). Data points represent the mean ± standard deviation. *: p value < 0.05 by *t*-test. (B) Cells were treated with osimertinib (O) and/or cetuximab (C). Data points represent the mean ± standard deviation. *: p value < 0.05 by *t*-test.

plates. The cells were treated with EGFR-TKIs (0.1 μM) and cetuximab (10 μg/mL) as a single or combination therapy for 48 h. The control cells were treated with the same concentration of the vehicle, DMSO. We analyzed the apoptotic status of cells using the Annexin V Apoptosis Detection Kit APC (eBioscience, San Diego, CA, USA), according to the manufacturer's protocol. The proportion of apoptotic cells was evaluated by flow cytometric analysis using the Gallios flow cytometer system (Beckman Coulter, Brea, CA, USA).

2.6. Mouse xenograft model

The animal experiment was approved by the Laboratory Animal

Table 1
IC_{50} values (nM) of afatinib/osimertinib alone or in combination with cetuximab.

Treatment	Exon 20 insertion mutations			
	ASV	HH	NPG	FQEA
Afatinib	72.6	166.6	27.9	0.8
Afatinib + Cetuximab (1 μg/mL)	7.9	58.8	9.2	0.7
Afatinib + Cetuximab (10 μg/mL)	13.2	32.9	9.0	0.6
Osimertinib	130.2	257.2	91.0	17.7
Osimertinib + Cetuximab (1 μg/mL)	40.2	113.6	87.8	5.8
Osimertinib + Cetuximab (10 μg/mL)	32.1	102.7	54.7	5.5

Center, Keio University School of Medicine. Female BALB/c-nu mice were purchased from Charles River (Kanagawa, Japan). The mice were anesthetized with ketamine, and 1 million Ba/F3 cells carrying *EGFR* A767_V769dupASV or Y764_V765insHH were injected subcutaneously as a Matrigel (Corning, NY, USA) suspension. When the average tumor volume reached approximately 180 mm^3, the mice were randomized to receive vehicle (control), afatinib (20 mg/kg, 5 days per week, orally), cetuximab (50 mg/kg, twice per week, intraperitoneally), and the combination of both. Subcutaneous tumors of these mice were monitored. The animals were humanely sacrificed and tumor tissues were harvested.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 4.0 (GraphPad Software, La Jolla, CA, USA). IC_{50} values were calculated using GraphPad Prism software. Student's *t* test was used for comparisons. All P values were two sided; values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Efficacy of afatinib or osimertinib plus cetuximab combination therapy on EGFR exon 20 insertion mutations

To evaluate the efficacy of cetuximab on *EGFR* exon 20 insertion mutations, we performed the MTS cell proliferation assay with cetuximab in a panel of Ba/F3 cells transduced with *EGFR* exon 20 insertion mutations (A767_V769dupASV, A764_V765insHH, D770_N771insNPG, and A763_Y764 in. FQEA). Except for *EGFR* A763_Y764insFQEA, an EGFR-TKI sensitive mutation, cetuximab did not induce an efficient inhibition of Ba/F3 cell proliferation (Fig. 1). These data indicated that cetuximab monotherapy has no effect on most *EGFR* exon 20 insertion mutations. Next, we evaluated the efficacy of afatinib or osimertinib plus cetuximab combination therapy on *EGFR* exon 20 insertion mutations. Notably, afatinib plus cetuximab combination therapy exerted an additional effect on A767_V769dupASV, A764_V765insHH, and D770_N771insNPG when compared with that of afatinib monotherapy (Fig. 2A). The difference of the additional effect was statistically significant.

Similarly, an additional effect was observed for osimertinib plus cetuximab combination therapy when compared with that of osimertinib monotherapy (Fig. 2B). The calculated IC_{50} values of these experiments are summarized in Table 1. In Ba/F3 cells expressing EGFR A767_V769dupASV, A764_V765insHH, or D770_N771insNPG, the IC_{50} values of afatinib or osimertinib used in combination with cetuximab were significantly lower than those of afatinib or osimertinib alone. However, no additional effect was observed using erlotinib in combination with cetuximab **(Supplementary Fig. S1)**. These data indicated

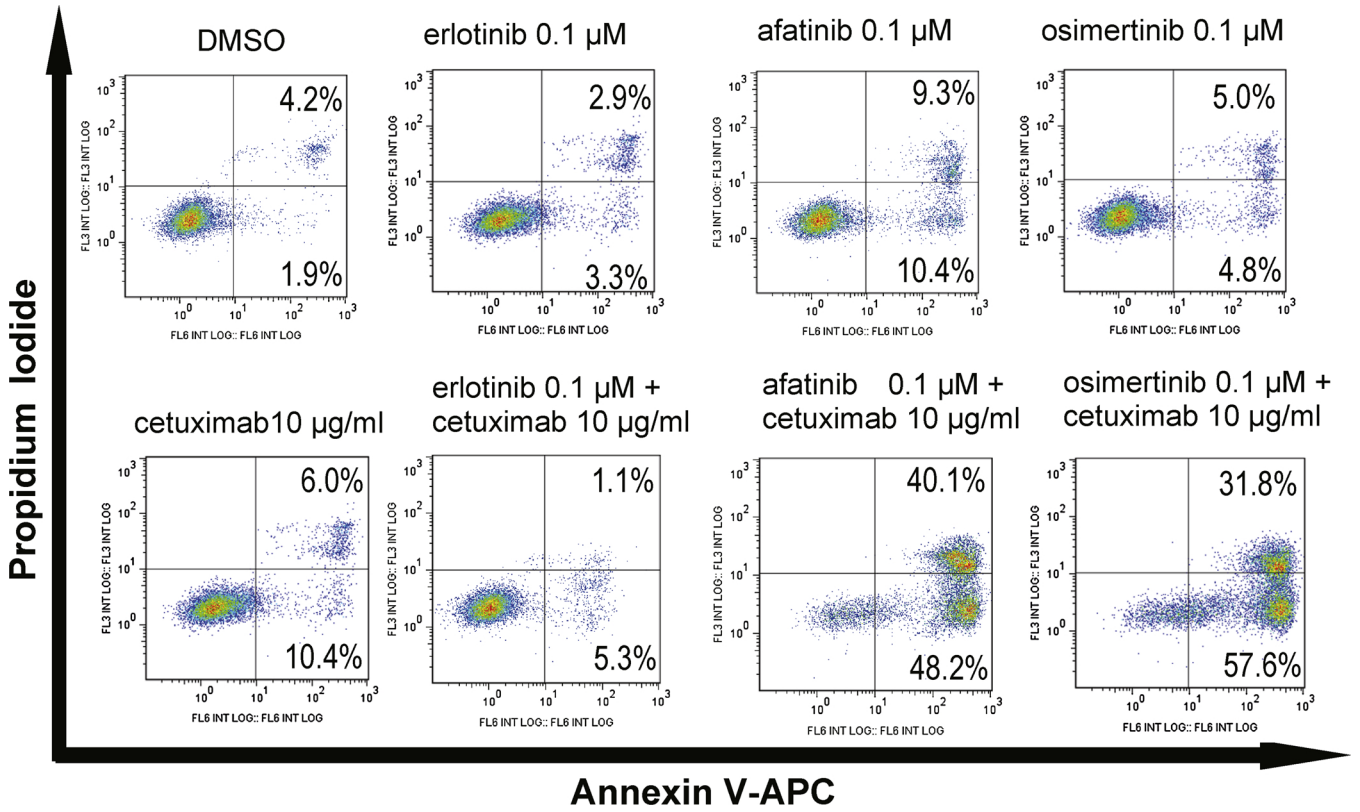

Fig. 3. Cetuximab enhanced apoptosis induced by afatinib or osimertinib in Ba/F3 cells harboring *EGFR* A767_V769dupASV.
Apoptosis assays of Ba/F3 cells harboring *EGFR* A767_V769dupASV were performed. The cells were treated with the indicated EGFR-TKI and/or cetuximab for 48 h prior to staining with propidium iodide and annexin V-APC for analysis by flow cytometry. The numbers indicate the percentages of cells in the annexin V- and/or propidium iodide-positive quadrants.

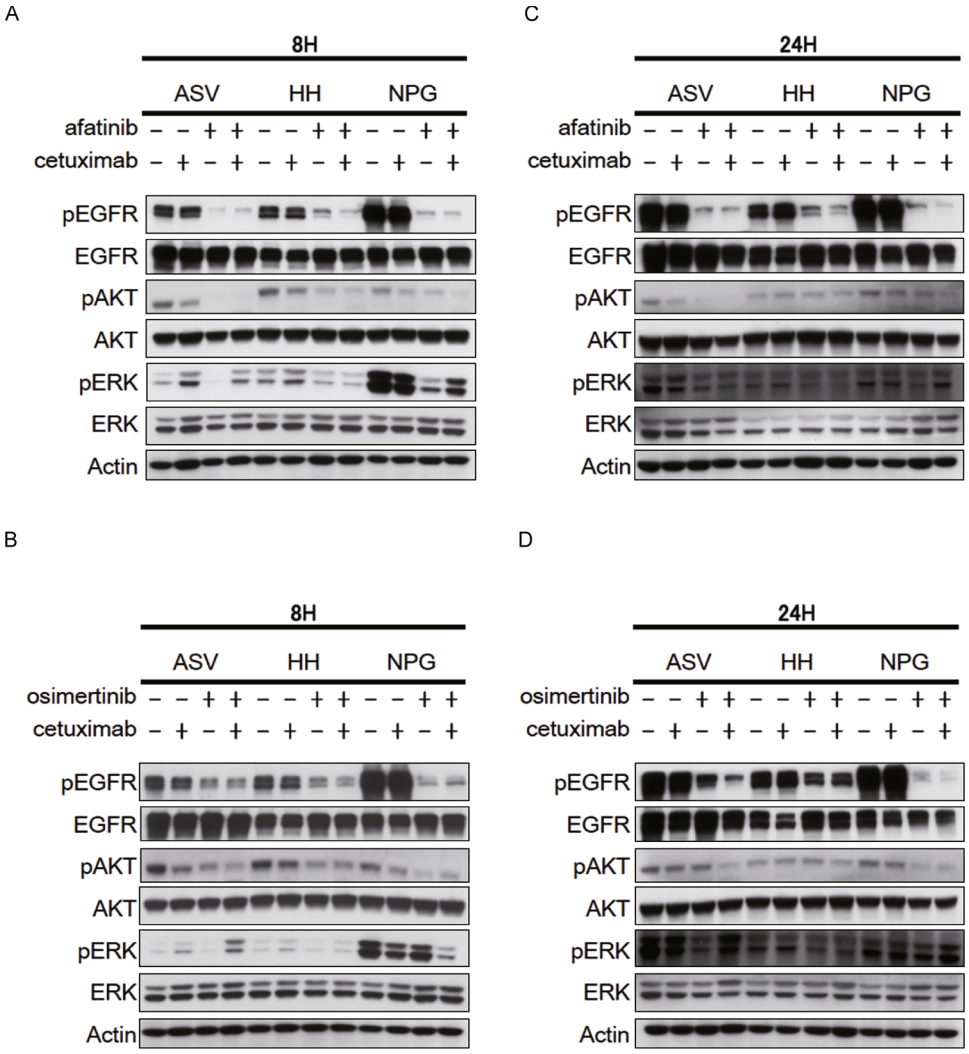

Fig. 4. Effects of EGFR-TKIs and/or cetuximab on signaling pathways downstream of EGFR in Ba/F3 cells expressing the indicated *EGFR* exon 20 insertions. Ba/F3 cells expressing the indicated *EGFR* exon 20 insertions were treated with EGFR-TKIs [afatinib (A and C) or osimertinib (B and D)] and/or cetuximab for 8 h or 24 h prior to immunoblotting for the phosphorylated forms of EGFR, AKT, and ERK. Actin was used as a loading control.

that afatinib or osimertinib plus cetuximab combination therapy is more effective than afatinib or osimertinib monotherapy.

3.2. Afatinib or osimertinib plus cetuximab combination therapy induces apoptosis in EGFR exon 20 insertion mutations

The observed efficacy of afatinib or osimertinib plus cetuximab combination therapy prompted us to examine whether the combination therapy could efficiently induce apoptosis in Ba/F3 cells carrying *EGFR* exon 20 insertion mutations. In Ba/F3 cells with A763_Y764insFQEA, all EGFR-TKIs, erlotinib, afatinib, and osimertinib, efficiently induced apoptosis as predicted **(Supplementary Fig. S2A)**. For *EGFR* A767_V769dupASV, one of the most frequent mutations among *EGFR* exon 20 insertion mutations, erlotinib, afatinib, or osimertinib did not efficiently induce apoptosis in cells carrying this mutation, and cetuximab monotherapy was also not active in this apoptosis test. Notably, afatinib plus cetuximab or osimertinib plus cetuximab combination therapy efficiently induced apoptosis in the cell lines carrying *EGFR* A767_V769dupASV, whereas erlotinib plus cetuximab combination therapy was not active in this assay (Fig. 3). Similarly, efficient induction of apoptosis by afatinib plus cetuximab combination therapy was observed in Ba/F3 cells with A764_V765insHH and D770_N771insNPG **(Supplementary Fig. S2B)**. These data demonstrated that afatinib or osimertinib plus cetuximab combination therapy induces apoptosis in *EGFR* exon 20 insertion mutations.

3.3. Inhibition of EGFR and downstream signaling pathways by afatinib or osimertinib plus cetuximab combination therapy

To verify that the inhibition of Ba/F3 cell proliferation was mediated by the inhibition of EGFR and the respective downstream signaling, we performed an immunoblot analysis on Ba/F3 cells carrying *EGFR* A767_V769dupASV, A764_V765insHH, or D770_N771insNPG. Although cetuximab monotherapy did not inhibit the phosphorylation of EGFR, AKT, and ERK after 8 h of treatment, afatinib and osimertinib monotherapies inhibited these phosphorylation effects **(**Fig. 4**A and B)**. The addition of cetuximab to afatinib or osimertinib slightly increased the inhibition of AKT phosphorylation. A similar inhibition profile was observed in Ba/F3 cells with *EGFR* mutations after 24 h of treatment **(**Fig. 4**C and D)**. Notably, the expression level of EGFR decreased in cetuximab-treated cells, demonstrating the effect of cetuximab on the degradation of EGFR protein. These data indicated that afatinib or osimertinib plus cetuximab combination therapy inhibited EGFR and its downstream signaling pathways.

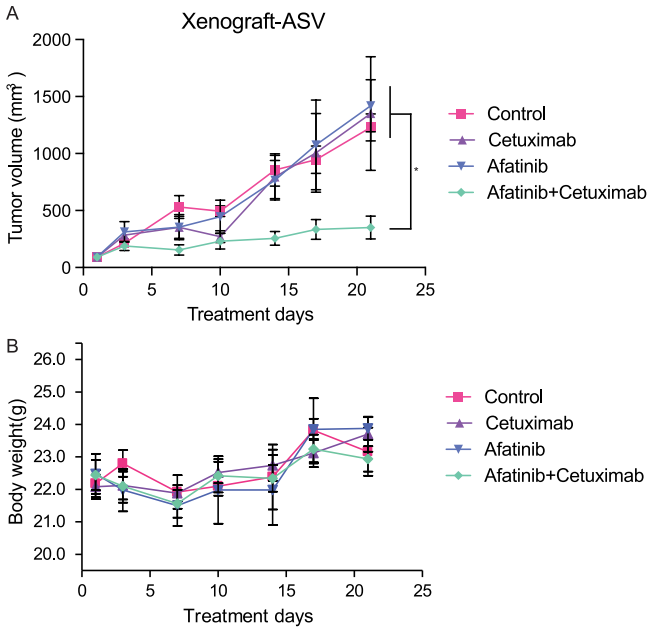

Fig. 5. Effect of afatinib and/or cetuximab on Ba/F3 cells expressing EGFR A767_V769dupASV *in vivo*.
(A) Ba/F3 tumor-bearing mice were randomly assigned to the control (n = 5), afatinib (n = 5), cetuximab (n = 5), or afatinib/cetuximab combination (n = 5) treatment group. Tumor size was measured to calculate tumor volume. Values indicate the average tumor volume in each group. *p < 0.05 by *t*-test for the afatinib/cetuximab combination group *versus* the control group or mice treated with either alone. Error bars indicate the standard deviation. (B) Body weights of the mice in each experimental group. Data points represent means ± standard deviation.

3.4. In vivo efficacy of afatinib plus cetuximab combination therapy on EGFR A767_V769dupASV

To confirm the efficacy of afatinib plus cetuximab combination therapy on *EGFR* exon 20 insertion mutations, we performed a xenograft experiment. Because Ba/F3 cells are pro-B cells whose growth depends on interleukin (IL)-3, we subcutaneously transplanted 1 million Ba/F3 cells carrying EGFR wildtype to nude mice to exclude the possibility of tumor growth depending on IL-3 or EGFR ligands expressed in mice subcutaneous microenvironment. However, no tumor growth was observed, indicating that the expression of IL-3 or EGFR ligands was not sufficient for Ba/F3 cells to form a tumor *in vivo* (data not shown). To examine whether Ba/F3 cells carrying *EGFR* A767_V769dupASV or Y764_V765insHH could form tumors *in vivo*, we transplanted these cells to the mice. Notably, the formation of tumors was observed for both cell lines, indicating that *EGFR* mutation-derived signals can induce tumor formation *in vivo*. After tumor formation, the mice were divided into four groups, namely control, cetuximab, afatinib, and afatinib plus cetuximab groups. Regarding *EGFR* A767_V769dupASV, although cetuximab and afatinib single treatment did not induce significant inhibition of tumor formation, afatinib plus cetuximab combination treatment induced a significant tumor growth inhibition ($p < 0.05$) **(**Fig. 5A**)**. In addition, a similar growth inhibition by the combination therapy was observed for *EGFR* Y764_V765insHH (**Supplementary Fig. S3A**). No significant body weight loss or skin rash was observed (Fig. 5B**, Supplementary Fig. S3B)**, indicating the tolerability of the combination therapy. Thus, our data demonstrated the *in vivo* efficacy of afatinib plus cetuximab combination therapy on several *EGFR* exon 20 insertion mutations.

4. Discussion

In this study, we evaluated the *in vitro and in vivo* efficacy of afatinib or osimertinib plus cetuximab combination therapy on *EGFR* exon 20 insertion mutations. An earlier report indicated a limited efficacy of 1st- and 2nd-generation EGFR-TKIs in NSCLC with *EGFR* exon 20 insertion mutations [19]. Therefore, NSCLC patients carrying these mutations cannot be effectively treated with EGFR-TKIs. Recently, we reported preclinical evidence for the potential efficacy of osimertinib on *EGFR* exon 20 insertion mutations [20]. To evaluate the efficacy of osimertinib in NSCLCs with *EGFR* exon 20 insertion mutations, multiple clinical trials (NCT03414814, NCT03191149) are being conducted including the AEX20 trial (UMIN000031929), for which we started recruitment in June 2018. Currently, the approved dose of osimertinib is 80 mg a day; however this dose may not be sufficient for demonstrating optimal effects against NSCLCs with these *EGFR* exon insertion mutations. The efficacy of poziotinib against cells carrying *EGFR* exon 20 insertion mutations has been reported [21]. TAS6417 was reported to be potentially effective in preclinical studies against cells with *EGFR* exon 20 insertions [22]. In addition to these inhibitors, TAK-788 has been developed recently as an EGFR exon 20 specific inhibitor, and its efficacy in clinical setting has been reported [23]. Furthermore, non-EGFR targeted therapies, such as heat shock protein 90 inhibitors, have demonstrated potential activity against *EGFR* exon 20 insertion mutations [24]. These data indicated the possibility that multiple inhibitors could become available for the treatment of NSCLCs with *EGFR* exon 20 insertion mutations. In our *in vitro* assays, afatinib or osimertinib plus cetuximab combination therapy induced an additive inhibitory effect on cell proliferation and downstream signaling and induced more profound levels of apoptosis in cells carrying several representative *EGFR* exon 20 insertion mutations. Notably, the inhibition of tumor growth *in vivo* occurred at a remarkable efficacy. These data indicate the possibility of the efficacy of the combination therapy against NSCLC with several of the known *EGFR* exon 20 insertion mutations. Although severe skin toxicities limit the application of this combination therapy in clinical setting [25], in support of our assumption, Veggel et al. recently reported the efficacy of afatinib and cetuximab combination therapy in three of four lung cancer patients with EGFR exon 20 insertion mutations [26]. In summary, we provide guidance based on promising preclinical results for using afatinib or osimertinib plus cetuximab combination therapy in NSCLC with *EGFR* exon 20 insertion mutations.

Funding

This work was supported in part by the Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to K.S. (15K09229), T.B. (15H04833), H.Y. (17K09667) and H.T. (18K08184). This work was supported in part by Takeda Science Foundation to H. Yasuda and H. Terai.

Conflicts of interest statement

There are no potential conflicts of interest to disclose.

Acknowledgements

We would like to thank Professor Tomoko Betsuyaku for financial assistance and technical advice. We are grateful to Ms. Mikiko Shibuya for her excellent technical assistance. We are also grateful to the Collaborative Research Resources at the Keio University School of Medicine for assistance with cell sorting.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2018.11.039.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2016, CA Cancer J. Clin. 66 (1) (2016) 7–30.

[2] N. Cancer Genome Atlas Research, comprehensive molecular profiling of lung adenocarcinoma, Nature 511 (7511) (2014) 543–550.

[3] T.J. Lynch, D.W. Bell, R. Sordella, S. Gurubhagavatula, R.A. Okimoto, B.W. Brannigan, P.L. Harris, S.M. Haserlat, J.G. Supko, F.G. Haluska, D.N. Louis, D.C. Christiani, J. Settleman, D.A. Haber, Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib, N. Engl. J. Med. 350 (21) (2004) 2129–2139.

[4] W. Pao, V. Miller, M. Zakowski, J. Doherty, K. Politi, I. Sarkaria, B. Singh, R. Heelan, V. Rusch, L. Fulton, E. Mardis, D. Kupfer, R. Wilson, M. Kris, H. Varmus, EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib, Proc. Natl. Acad. Sci. U. S. A. 101 (36) (2004) 13306–13311.

[5] S.V. Sharma, D.W. Bell, J. Settleman, D.A. Haber, Epidermal growth factor receptor mutations in lung cancer, Nat. Rev. Cancer 7 (3) (2007) 169–181.

[6] C.H. Yun, T.J. Boggon, Y. Li, M.S. Woo, H. Greulich, M. Meyerson, M.J. Eck, Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity, Cancer Cell 11 (3) (2007) 217–227.

[7] N. Jura, X. Zhang, N.F. Endres, M.A. Seeliger, T. Schindler, J. Kuriyan, Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms, Mol. Cell 42 (1) (2011) 9–22.

[8] X. Zhang, J. Gureasko, K. Shen, P.A. Cole, J. Kuriyan, An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor, Cell 125 (6) (2006) 1137–1149.

[9] D.B. Costa, B. Halmos, A. Kumar, S.T. Schumer, M.S. Huberman, T.J. Boggon, D.G. Tenen, S. Kobayashi, BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations, PLoS Med. 4 (10) (2007) 1669–1679 discussion 1680..

[10] T.S. Mok, Y.L. Wu, S. Thongprasert, C.H. Yang, D.T. Chu, N. Saijo, P. Sunpaweravong, B. Han, B. Margono, Y. Ichinose, Y. Nishiwaki, Y. Ohe, J.J. Yang, B. Chewaskulyong, H. Jiang, E.L. Duffield, C.L. Watkins, A.A. Armour, M. Fukuoka, Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma, N. Engl. J. Med. 361 (10) (2009) 947–957.

[11] M. Maemondo, A. Inoue, K. Kobayashi, S. Sugawara, S. Oizumi, H. Isobe, A. Gemma, M. Harada, H. Yoshizawa, I. Kinoshita, Y. Fujita, S. Okinaga, H. Hirano, K. Yoshimori, T. Harada, T. Ogura, M. Ando, H. Miyazawa, T. Tanaka, Y. Saijo, K. Hagiwara, S. Morita, T. Nukiwa, G. North-East Japan Study, Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR, N. Engl. J. Med. 362 (25) (2010) 2380–2388.

[12] T. Mitsudomi, S. Morita, Y. Yatabe, S. Negoro, I. Okamoto, J. Tsurutani, T. Seto, M. Satouchi, H. Tada, T. Hirashima, K. Asami, N. Katakami, M. Takada, H. Yoshioka, K. Shibata, S. Kudoh, E. Shimizu, H. Saito, S. Toyooka, K. Nakagawa, M. Fukuoka, G. West Japan Oncology, 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, The Lancet, Oncology 11 (2) (2010) 121–128.

[13] C. Zhou, Y.L. Wu, G. Chen, J. Feng, X.Q. Liu, C. Wang, S. Zhang, J. Wang, S. Zhou, S. Ren, S. Lu, L. Zhang, C. Hu, C. Hu, Y. Luo, L. Chen, M. Ye, J. Huang, X. Zhi, Y. Zhang, Q. Xiu, J. Ma, L. Zhang, C. You, 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, the Lancet, Oncology 12 (8) (2011) 735–742.

[14] Y.L. Wu, Y. Cheng, X. Zhou, K.H. Lee, K. Nakagawa, S. Niho, F. Tsuji, R. Linke, R. Rosell, J. Corral, M.R. Migliorino, A. Pluzanski, E.I. Sbar, T. Wang, J.L. White, S. Nadanaciva, R. Sandin, T.S. Mok, Dacomitinib versus gefitinib as first-line treatment for patients with EGFR-mutation-positive non-small-cell lung cancer (ARCHER 1050): a randomised, open-label, phase 3 trial, The Lancet, Oncology (2017).

[15] L.V. Sequist, J.C. Yang, N. Yamamoto, K. O'Byrne, V. Hirsh, T. Mok, S.L. Geater, S. Orlov, C.M. Tsai, M. Boyer, W.C. Su, J. Bennouna, T. Kato, V. Gorbunova, K.H. Lee, R. Shah, D. Massey, V. Zazulina, M. Shahidi, M. Schuler, Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations, J. Clin. Oncol. 31 (27) (2013) 3327–3334.

[16] S.S. Ramalingam, J.C. Yang, C.K. Lee, T. Kurata, D.W. Kim, T. John, N. Nogami, Y. Ohe, H. Mann, Y. Rukazenkov, S. Ghiorghiu, D. Stetson, A. Markovets, J.C. Barrett, K.S. Thress, P.A. Janne, Osimertinib As First-Line Treatment of EGFR Mutation-Positive Advanced Non-Small-Cell Lung Cancer, J. Clin. Oncol. (2017) JCO2017747576.

[17] S. Kobayashi, T.J. Boggon, T. Dayaram, P.A. Janne, O. Kocher, M. Meyerson, B.E. Johnson, M.J. Eck, D.G. Tenen, B. Halmos, EGFR mutation and resistance of non-small-cell lung cancer to gefitinib, N. Engl. J. Med. 352 (8) (2005) 786–792.

[18] H. Yasuda, E. Park, C.H. Yun, N.J. Sng, A.R. Lucena-Araujo, W.L. Yeo, M.S. Huberman, D.W. Cohen, S. Nakayama, K. Ishioka, N. Yamaguchi, M. Hanna, G.R. Oxnard, C.S. Lathan, T. Moran, L.V. Sequist, J.E. Chaft, G.J. Riely, M.E. Arcila, R.A. Soo, M. Meyerson, M.J. Eck, S.S. Kobayashi, D.B. Costa, Structural, Biochemical, and Clinical Characterization of Epidermal Growth Factor Receptor (EGFR) Exon 20 Insertion Mutations in Lung Cancer, Sci. Transl. Med. 5 (216) (2013).

[19] H. Yasuda, S. Kobayashi, D.B. Costa, EGFR exon 20 insertion mutations in non-small-cell lung cancer: preclinical data and clinical implications, the Lancet, Oncology 13 (1) (2012) e23–31.

[20] T. Hirano, H. Yasuda, T. Tani, J. Hamamoto, A. Oashi, K. Ishioka, D. Arai, S. Nukaga, M. Miyawaki, I. Kawada, K. Naoki, D.B. Costa, S.S. Kobayashi, T. Betsuyaku, K. Soejima, In vitro modeling to determine mutation specificity of EGFR tyrosine kinase inhibitors against clinically relevant EGFR mutants in non-small-cell lung cancer, Oncotarget 6 (36) (2015) 38789–38803.

[21] J.P. Robichaux, Y.Y. Elamin, Z. Tan, B.W. Carter, S. Zhang, S. Liu, S. Li, T. Chen, A. Poteete, A. Estrada-Bernal, A.T. Le, A. Truini, M.B. Nilsson, H. Sun, E. Roarty, S.B. Goldberg, J.R. Brahmer, M. Altan, C. Lu, V. Papadimitrakopoulou, K. Politi, R.C. Doebele, K.K. Wong, J.V. Heymach, Mechanisms and clinical activity of an EGFR and HER2 exon 20-selective kinase inhibitor in non-small cell lung cancer, Nat. Med. 24 (5) (2018) 638–646.

[22] S. Hasako, M. Terasaka, N. Abe, T. Uno, H. Ohsawa, A. Hashimoto, R. Fujita, K. Tanaka, T. Okayama, R. Wadhwa, K. Miyadera, Y. Aoyagi, K. Yonekura, K. Matsuo, TAS6417, A Novel EGFR Inhibitor Targeting Exon 20 Insertion Mutations, Mol. Cancer Ther. 17 (8) (2018) 1648–1658.

[23] R.C. Doebele, G.J. Riely, A.I. Spira, L. Horn, Z. Piotrowska, D.B. Costa, First report of safety, PK, and preliminary antitumor activity of the oral EGFR/HER2 exon 20 inhibitor TAK-788 (AP32788) in non-small cell lung cancer (NSCLC), J. Clin. Oncol. 36 (15) (2018).

[24] S.E. Jorge, A.R. Lucena-Araujo, H. Yasuda, Z. Piotrowska, G.R. Oxnard, D. Rangachari, M.S. Huberman, L.V. Sequist, S.S. Kobayashi, D.B. Costa, EGFR Exon 20 Insertion Mutations Display Sensitivity to Hsp90 Inhibition in Preclinical Models and Lung Adenocarcinomas, Clin. Cancer Res. (2018).

[25] Y.Y. Janjigian, E.F. Smit, H.J. Groen, L. Horn, S. Gettinger, D.R. Camidge, G.J. Riely, B. Wang, Y. Fu, V.K. Chand, V.A. Miller, W. Pao, Dual inhibition of EGFR with afatinib and cetuximab in kinase inhibitor-resistant EGFR-mutant lung cancer with and without T790M mutations, Cancer Discov. 4 (9) (2014) 1036–1045.

[26] B. van Veggel, A.J. de Langen, S.M.S. Hashemi, K. Monkhorst, D.A.M. Heideman, E. Thunnissen, E.F. Smit, Afatinib and cetuximab in four patients with EGFR exon 20 insertion-positive advanced NSCLC, J. Thorac. Oncol. 13 (8) (2018) 1222–1226.