博 士 学 位 論 文

一次治療オシメルチニブに対する耐性化二次変異 in vitro model の検討

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Doctoral Dissertation

Effects of secondary *EGFR* mutations on resistance against upfront osimertinib in cells with *EGFR*-activating mutations *in vitro*

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論文題目
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Effects of secondary *EGFR* mutations on resistance against upfront osimertinib in cells with *EGFR*-activating mutations *in vitro*

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Abstract

Objectives: Non-small cell lung cancers (NSCLCs) that harbor activating mutations for epidermal growth factor receptor (*EGFR*) show remarkable initial response to EGFR-tyrosine kinase inhibitors (TKIs), but inevitably acquire resistance, half of which are due to a T790M secondary mutation when first-generation (1G) or 2G EGFR-TKIs are used. Osimertinib, a 3G EGFR-TKI, is a standard of care in this situation, but eventually also evokes resistance, reportedly due to some tertiary *EGFR* mutations. However, the FLAURA trial showed the superiority of osimertinib over 1G EGFR-TKIs in treatment-naïve patients, thus providing an option of first-line osimertinib treatment. Resistance in this setting is also inevitable, but its mechanism is unclear. We investigated whether resistance mutations that emerged with T790M were responsible for the osimertinib resistance in the first-line setting; i.e. without T790M, and if so, what treatment option was available.

Materials and Methods: We used literature search to identify *EGFR* mutations at codons L718, G724, L792, G796, and C797 as mechanisms of osimertinib resistance in the presence of T790M. These mutations were introduced into Ba/F3 cells in *cis* with activating *EGFR* mutations but not with T790M; inhibitory effects of five EGFR-TKIs were evaluated.

Results: Only C797S conferred significant resistance against osimertinib when exon 19 deletion was the activating mutation. However, co-existence of L858R with C797S, C797G, L718Q, or L718V mutations all conferred resistance to osimertinib. Erlotinib showed the greatest activity for C797S-mediated resistance. However, 2G EGFR-TKIs (afatinib or dacomitinib) were effective for other resistance mutations.

Conclusion: After first-line osimertinib failure, 1G or 2G EGFR-TKIs are effective, depending on combinations of secondary and activating mutations.

Key words : epidermal growth factor receptor mutations, osimertinib, acquired resistance, Ba/F3 models, personalized therapy

1. Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) monotherapy is the current standard of care for patients with metastatic or recurrent non-small cell lung cancer (NSCLC) with *EGFR*-activating mutations. However, the emergence of acquired resistance is almost inevitable after a median period of 9–13 months [1]. The T790M secondary mutation [2-4] accounts for about half of resistance to first-generation (1G) or the 2G EGFR-TKIs. Osimertinib, a so-called 3G EGFR-TKI, was developed to inhibit T790M while sparing wild-type EGFR activity [5]. Osimertinib is currently a standard of care for *EGFR*-mutated NSCLCs with acquired resistance to 1G or 2G EGFR-TKIs due to the T790M mutation, based on the AURA 3 trial [5]. However, acquired resistance to osimertinib is also inevitable. Several tertiary *EGFR* mutations—including C797S mutation, which impairs covalent bond formation between EGFR and osimertinib—have been reported [6].

In a recent phase-3 FLAURA trial [7], osimertinib led to significantly longer progression-free survival and promising overall survival than did 1G EGFR-TKIs as a first-line treatment for NSCLC patients with activating *EGFR* mutations. These results provide another option for the first-line treatment of patients with *EGFR*-activating mutations. However, acquisition of resistance in this setting is also inevitable. Some of above-mentioned tertiary *EGFR* mutations may have roles in this resistance, but have not been systematically tested. In this study, we investigated whether resistance mutations that emerged with T790M were also responsible for osimertinib resistance in the first-line setting (i.e., without T790M) using the Ba/F3 system, and if so, what treatment option was available after osimertinib treatment failure.

2. Materials and Methods

2. 1 Cell culture and reagents

The murine pro-B cell line Ba/F3 (RCB0805) and myelomonocytic, macrophage-like, Balb/C mouse leukemia cells (WEHI-3) were provided by the RIKEN Bio Resource Center (Tsukuba, Japan). The Ba/F3 cells were maintained in RPMI 1640 medium (Wako, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY) and conditioned media from WEHI-3 (10%) as a source of IL-3, and cultured at 37℃ in a humid atmosphere with 5% CO_2 [8]. We purchased 1G EGFR-TKI (erlotinib), 2G EGFR-TKIs (afatinib and dacomitinib), 3G EGFR-TKI (osimertinib), and a multi-kinase inhibitor that is also active against EGFR (brigatinib, ref. [9]) from Selleck Chemicals (Houston, TX). Each compound was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO).

2. 2 Plasmid construction and viral production

Viral particles that contained human *EGFR* open-reading frame with an activating mutation (either exon 19 deletion (Del 19) or L858R) plus a potential osimertinib resistant mutation were generated as described previously [10]. To establish the viral particles, pBABE with a full-length cDNA fragment of human *EGFR*

with Del 19 (E746_A750 del) and that with Del 19 plus T790M were purchased from Addgene (Cambridge, MA) as a template. For the viral particles with L858R mutation plus a point mutation, pBABE with a full-length wild-type *EGFR* cDNA fragment (Addgene) was used. The intended point mutations were induced using the Prime STAR Mutagenesis Basal Kit (Takara, Otsu, Japan). Sequences for primers that were used for mutagenesis PCRs in this study are summarized in Supplementary Table 1. All mutations were confirmed by direct sequencing technique as described elsewhere [10, 11]. The pBABE constructs were co-transfected with a pVSV-G vector (Clontech, Mountain View, CA) to generate the viral envelope in gpIRES-293 cells using the FuGENE6 transfection reagent (Promega, Madison, Wisconsin) to produce viral particles. After 48h of transfection, the culture medium was collected, and the viral particles were concentrated by centrifugation. The viral pellet was re-suspended in DMEM (Sigma-Aldrich) and stored at −80℃.

Table 1. Summary of tertiary *EGFR* mutations found in osimertinib-resistant tumors

Mutations [references]	Type of background activating mutation				Ba/F3 clones established in our experiment	
	Del 19 / T790M	L858R / T790M	Others / T790M	Total	Del 19	L858R
L718Q [12, 15, 20]	3 (6%)*	4 (16%)*	0 (0%)	7 (9%)		Y
L718V [13, 20]	2 (4%)	3 (12%)	0 (0%)	5 (7%)	Y	Y
G724S [14]	2 (4%)*	0 (0%)	0 (0%)	2 (3%)	Y	
L792F [15, 16, 20]	7 (15%)	3 (12%)	1 (25%)	11 (14%)	Y	Y
L792H [15, 16, 20]	4 (9%)	3 (12%)	1 (25%)	8 (11%)	Y	Y
G796S [16, 17, 20]	1 (2%)	3 (12%)	0 (0%)	4 (5%)		
C797G [15, 16, 18, 20]	4 (9%)	2 (8%)	1 (25%)	7 (9%)	**	Y
C797S [6, 15, 16, 19, 20]	24 (51%)	7 (28%)	1 (25%)	32 (42%)	Y	Y
Total	47 (100%)	25 (100%)	4 (100%)	76 (100%)		

* Two patients with exon 19 deletions acquired either L718Q or G724S mutations as secondary mutations, and two patients with L858R acquired L718Q mutations as secondary mutations after osimertinib treatment failure. The other tumors harbored T790M mutations together with a tertiary *EGFR* mutation

** Although an IL-3 independent clone was established, the clone acquired an additional mutation to cause C797D mutation.

2. 3 Viral infection into Ba/F3 cells and the evaluation of IL-3 independency

Re-suspended viral particles were added to Ba/F3 cells; the infected Ba/F3 cells were then cultured with conditioned media that contains IL-3. After 72 hours, the infected Ba/F3 cells were cultured without IL-3 for another 96 hours, and then cell growth were analyzed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) as described below. Infected Ba/F3 cells that acquired IL-3 independency were used for further analyses. Ba/F3 cells transfected with some of the *EGFR* mutations did not acquire IL-3 independent

growth, and this result was confirmed by repeated experiments using newly generated viral particles.

2. 4 Cell growth inhibition assay

We seeded 2000 transfected Ba/F3 cells in each well of 96-well plates, which were grown in RPMI 1640 medium supplemented with 10% FBS for 24 hours. Then DMSO or TKIs at indicated drug concentrations were added, and the cells were cultured for another 72 hours. We used colorimetric assays to estimate the growth inhibition of each drug using the Cell Counting Kit-8 reagent (Dojindo Laboratories), following the manufacturer's protocol. Each experiment was performed three times in triplicate.

3. Results

3. 1 Identification of EGFR mutations that might confer osimertinib resistance

We identified eight recurrent *EGFR* tertiary mutations that may confer osimertinib resistance in the presence of T790M through a literature search (Table 1)[6, 12–20]. C797S was the most common mutation (51% in tumors with Del 19 and 28% in those with L858R). In addition, point mutations in the L718 position (L718Q/V) or in the L792 position (L792F/H) were relatively common, especially in tumors with L858R mutation. We decided to evaluate the effects of these eight point mutations on sensitivity of Ba/F3 cells with Del 19 or L858R to osimertinib and other EGFR-TKIs.

3. 2 Some resistant mutations were not compatible with activating mutations

Although we could introduce eight resistant mutations together with Del 19 or L858R into Ba/F3 cells (Supplementary Figure 1), some did not become IL-3-independent. These Ba/F3 cells that failed to establish IL-3 independent growth were confirmed to have been successfully transfected by direct sequencing in the presence of IL-3. Those included G796S+Del 19 or L858R, L718Q+Del 19, and G724S+L858R despite repeated independent experiments (Figure 1A). In the case of C797G+Del 19, although we finally established an IL-3-independent clone after a long-term culture, this clone turned out to have C797D instead. As shown in Figure 1B, the second nucleotide of CGC for glycine (G) was replaced with A to become GAC for aspartic acid (D). The Ba/F3 cells with Del 19+C797D showed resistance to osimertnib (IC_{50}: 695nM). This phenomenon was reproducible three times. As a result, we decided to test the 5 resulting mutations (L718V, G724S, L792F, L792H, C797S) +Del 19 and 6 mutations (L718Q, L718V, L792F, L792H, C797G, C797S) +L858R for TKI sensitivities.

3. 3 Effects of secondary mutations on osimertinib sensitivity

To determine the effect of each point mutation on osimertinib efficacy, growth inhibitory assays were performed for the Ba/F3 cells. When introduced together with Del 19, C797S was the only secondary mutation that conferred significant resistance to osimertinib (IC_{50} > 50 times higher than the activating

mutation alone; Figure 1C). Ba/F3 cells remained relatively sensitive when other secondary mutations were introduced together with Del 19 and its IC_{50} values were far below than C_{Trough} at the recommended dose in the phase I clinical trials [21－25]. In contrast, C797S, C797G, L718Q, and L718V mutations conferred >100 times higher IC_{50} value when they were transfected with L858R (Figure 1D). In addition, L792F and L792H also conferred moderate (>20 times) resistance if the activating mutation was L858R.

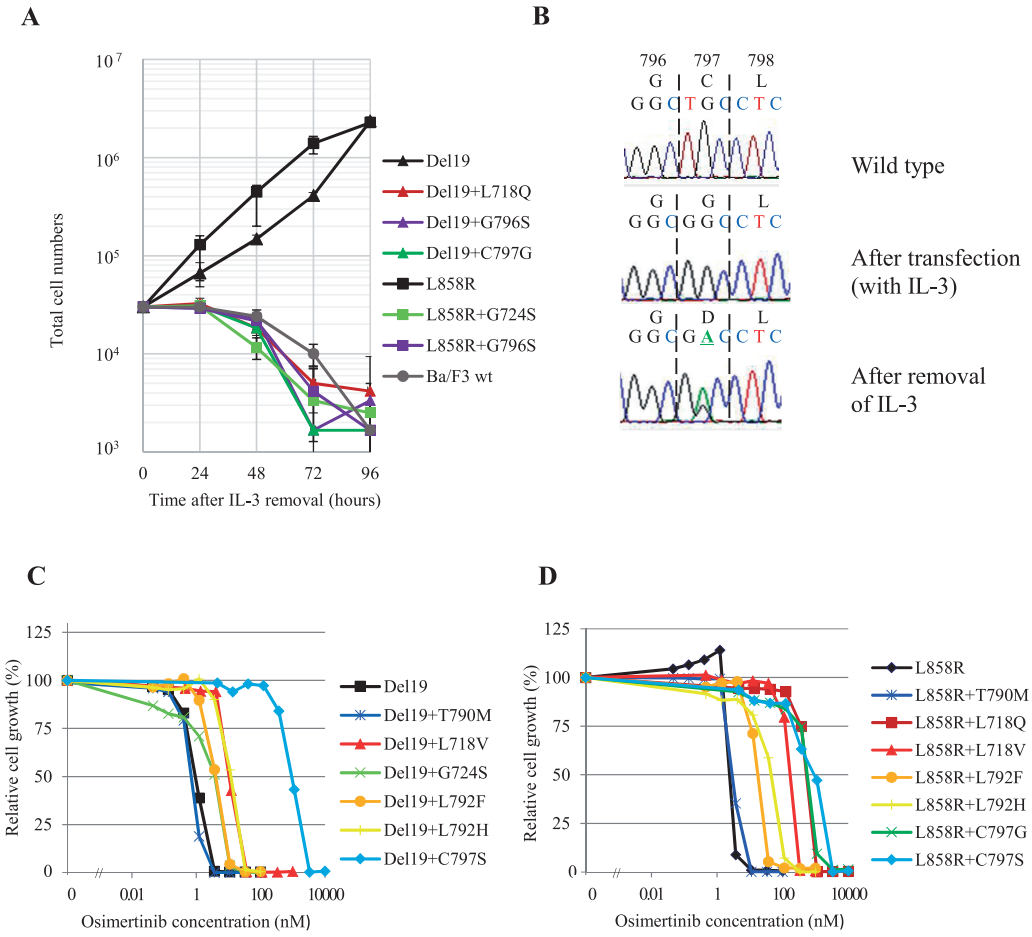


Figure 1. Establishment of osimertinib-resistant Ba/F3 cells and evaluation of sensitivity to osimertinib. (A) Growth curves of transfected Ba/F3 cells after IL-3 starvation. The graph shows that Ba/F3 cells transfected with exon 19 deletion + L718Q, G796S, or C797G, and those transfected with L858R + G724S or G796S failed to become IL-3-independent. (B) Although Ba/F3 cells transfected with exon 19 deletion + C797G acquired IL-3 independency after long culture, the established cells acquired C797D mutation. (C, D) Growth inhibition curves against osimertinib in Ba/f3 cells transfected with exon 19 deletion + a point mutation (C) and in those transfected with L858R + a point mutation (D).

3. 4 Optimal EGFR-TKIs after osimertinib failure are based on the type of EGFR-activating mutations.

To explore optimal treatment strategy to cope with resistance mediated by the above-mentioned secondary mutations, we determined their sensitivities to 1G (erlotinib), 2G EGFR-TKIs (afatinib and dacomitinib), and

a multi-kinase inhibitor (brigatinib). When Del 19 was the background mutation, erlotinib was efficacious for C797S (IC_{50} < 0.1% of C_{Trough}). However, dacomitinib or afatinib was moderately efficacious for C797S. Interestingly, Del 19 + G724S was even more sensitive than Del 19 alone to afatinib or dacomitinib (Figure 2). For Ba/F3 cells with L858R mutation, erlotinib also showed greatest efficacy against C797S secondary mutation (Figure 3). However, for the other secondary mutations, 2G EGFR-TKIs, especially dacomitinib, showed greater efficacy than erlotinib. Brigatinib was ineffective for any of these secondary mutations (Figures 2 and 3).

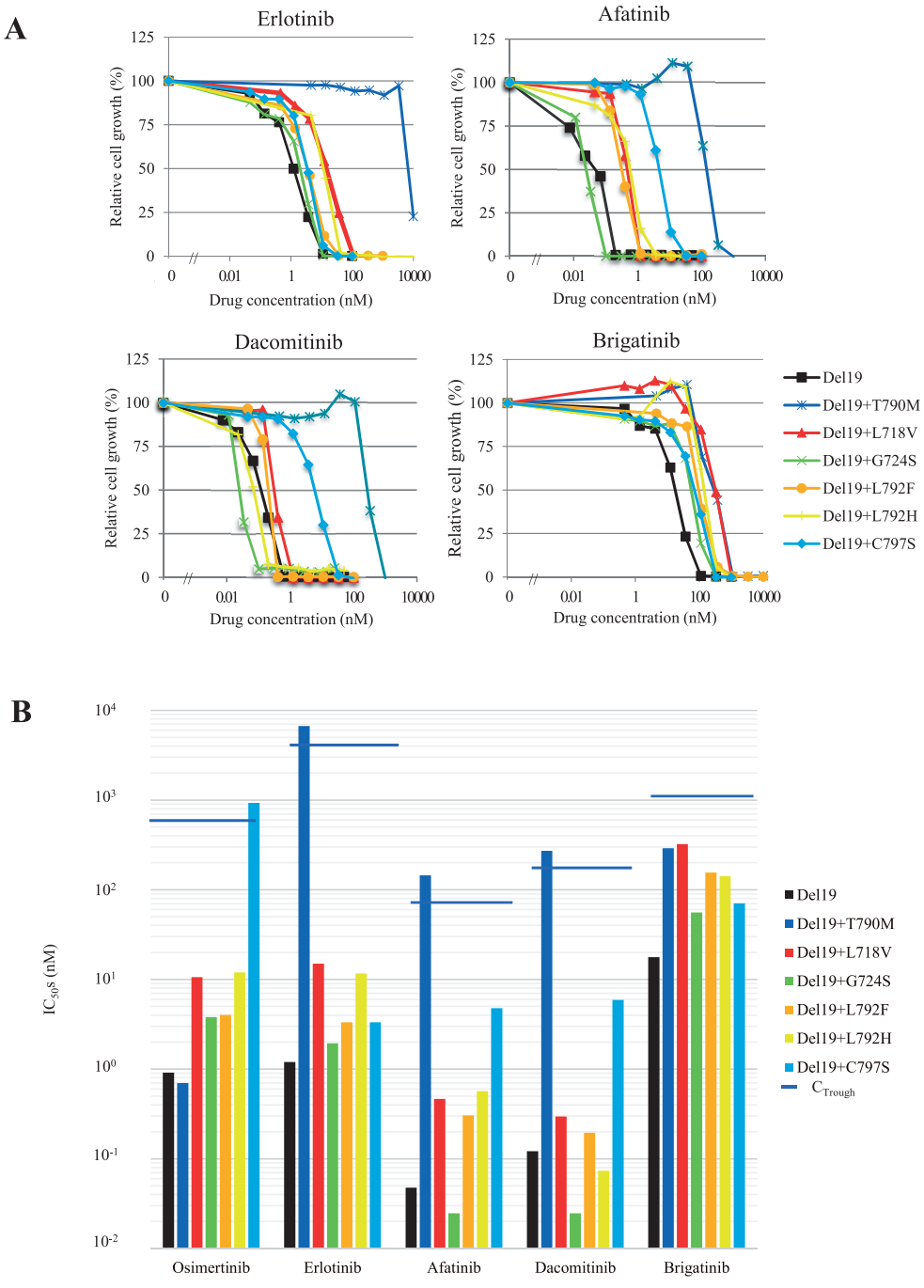


Figure 2. EGFR-TKI sensitivities in transfected Ba/F3 cells (activating mutation: exon 19 deletion). (A) Growth inhibition curves against erlotinib, afatinib, dacomitinib, or brigatinib. (B) Comparison of C_{Trough} of each TKI and IC_{50} of transfected Ba/F3 cells.

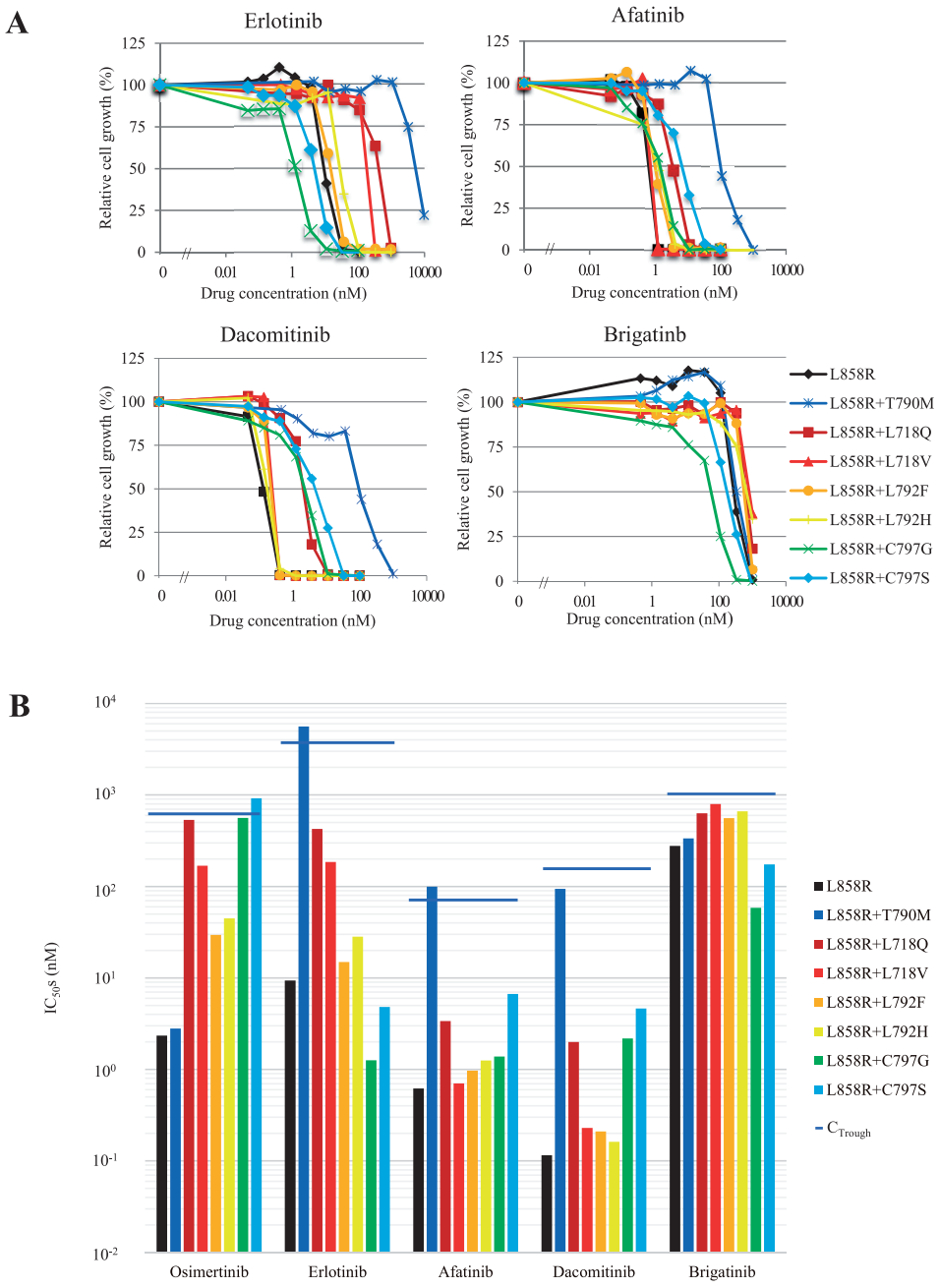


Figure 3. EGFR-TKI sensitivities in transfected Ba/F3 cell (activating mutation: L858R). (A) Growth inhibition curves against erlotinib, afatinib, dacomitinib, or brigatinib. (B) Comparison of C_{Trough} of each TKI and IC_{50} of transfected Ba/F3 cells.

4. Discussion

In this study, we introduced potential osimertinib-resistance point mutations reported in the literature with Del 19 or L858R into Ba/F3 cells, to evaluate the magnitude of osimertinib resistance and to identify optimal EGFR-TKIs to overcome osimertinib resistance. Our experiments were based on the assumption that the presence of T790M mutation will not affect the binding of osimertinib to EGFR. Our results indicated that our

assumption was in fact correct; many of the tertiary mutations identified in the second-line osimertinib setting conferred resistance to osimertinib even as a secondary mutation (Figure 1C and D). We found that C797S was the most potent resistant mutation against osimertinib in the Del 19 background. Other mutations only conferred modest resistance. In contrast, L718Q, L718V, C797G conferred comparable resistance with C797S in the L858R background. These results are consistent with the clinical observation that the prevalence of C797S accounted for more than half of resistance mutations in the Del 19 background, whereas L718, L792, and C797 were almost equally common in the L858R background (Table 1). Previously, we proposed that the extent of resistance was determined by combinations of the original mutations, secondary mutations, and drugs, using an afatinib resistance system [26]. For example, a very sensitive *EGFR* mutation (e.g., Del 19) needs a robust resistant secondary mutation (T790M), whereas a weaker secondary mutations (L792F) could confer resistance for the weaker sensitizing mutation, delE709_T710insD [26]. This is mirrored in our current results regarding osimertinib resistance. Together with a recent observation that median progression-free survival is longer in patients with Del 19 mutations (21 months) than those with L858R mutations (14 months) in the osimertinib FLAURA trial [7], we consider that the current study also supports our previous proposal. I.e., a robust resistance mutation (C797S) is necessary for Del 19, whereas weaker secondary mutations are able to cause resistance when the activating mutation is L858R.

Among the 8 mutations that were reported to yield osimertinib resistance as tertiary mutations in addition to Del19 or L858R +T790M, five mutations did not yield IL-3-independent growth despite repeated experiments. The reason for this may be related to the fact that T790M was not present in our experiments because we intended to simulate the situation of first-line osimertinib treatment. For example, we could not obtain IL-3-independent Ba/F3 cells with Del 19 + L718Q. In a previous study [27], Ercan, et al. derived Ba/F3 cells resistant to 3G EGFR-TKIs, including osimertinib, by ENU mutagenesis and L718Q was one of the resistant mutations in the background of Del 19 with T790M, but not of Del 19 without T790M. In contrast, L718Q conferred resistance to L858R irrespective of presence or absence of T790M with higher frequency in the L858R-alone background than in the L858R+T790M background when treated with another 3G TKI, WZ4002. To evaluate this hypothesis, we generated templates with T790M mutation together with one of five double EGFR mutations that could not confer IL-3 independent growth in our Ba/F3 models. We observed that Ba/F3 cells with Del 19 + T790M + L718Q and Del 19 + T790M + C797G acquired IL-3 independent growth, while the other three combinations failed. These results suggested that our hypothesis is partially correct.

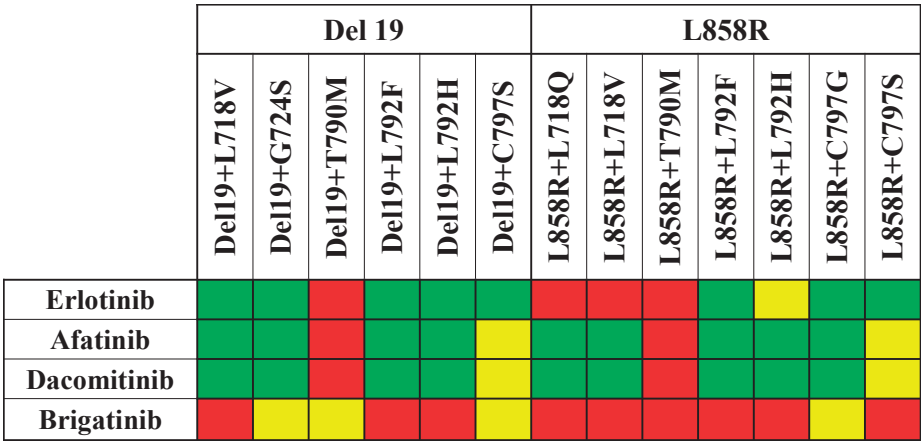
Taken together, these findings indicate some interactions between amino acids within the EGFR molecule. Hence, some resistant mutations that are compatible with Del 19 + T790M may not be compatible with Del 19 alone. Notably, in this context, Ba/F3 cells with Del19+ C797G acquired an additional spontaneous base substitution to result in C797D, when they become IL-3-independent (Figure 1B).

We also observed that sensitivity to 1G or 2G EGFR-TKIs varied by both the secondary mutations and by

the type of activating mutations, highlighting the importance of their combination. We showed that the cells with C797S + Del 19 or L858R were sensitive to 1G EGFR-TKI, erlotinib. However, afatinib and dacomitinib showed greater efficacy than erlotinib on resistance mutations other than C797S/G in the L858R background. These results were reasonable, since 2G EGFR-TKIs covalently bind to EGFR via C797, however 2G EGFR-TKIs are active against wide range of *EGFR* mutations including uncommon mutations [28].

Based on our results considering the C_{Trough} of each drug, we propose that erlotinib be given when secondary mutations are present in the Del 19 background, and afatinib or dacomitinib be given when secondary mutations other than C797S/G are present in the L858R background (Figure 4). Although brigatinib combined with anti-EGFR antibody was effective in mice model with *EGFR* activating mutation + T790M + C797S [9], our results indicated that brigatinib monotherapy could not overcome any secondary mutations after front-line osimertinib .

In conclusion, our results suggest that the effects of secondary mutations as osimertinib resistant mechanisms will differ by the type of activating *EGFR* mutations. 1G and/or 2G EGFR-TKIs will be effective after first-line osimertinib treatment failure, and their efficacy will be affected by both secondary point mutations and the type of activating *EGFR* mutations.



	Erlotinib	Afarinib/Dacomitinib	Osimertinib	Brigatinib
Sensitive	$IC_{50} \leqq 20nM$	$IC_{50} \leqq 4.5nM$	$IC_{50} \leqq 10nM$	$IC_{50} \leqq 20nM$
Intermediate	$20nM < IC_{50} \leqq 100nM$	$4.5nM < IC_{50} \leqq 10nM$	$10nM < IC_{50} \leqq 50nM$	$20nM < IC_{50} \leqq 100nM$
Resistant	$100nM < IC_{50}$	$10nM < IC_{50}$	$50nM < IC_{50}$	$100nM < IC_{50}$

Figure 4. Summary of sensitivities against various TKIs in our Ba/F3 models. Each mutation is classified as sensitive, intermediate or resistant to each drug.

Disclosure of Potential Conflicts of Interest

Y. Kobayashi has received honoraria from Boehringer Ingelheim. T. Mitsudomi has received honoraria from AstraZeneca, Boehringer Ingelheim, Chugai, and Pfizer, has played a role for consultant/advisory board

member for AstraZeneca, Chugai, and Boehringer Ingelheim, and has received research funding (through Kindai University Faculty of Medicine) from AstraZeneca, Boehringer Ingelheim, and Chugai. The other authors declare no potential conflicts of interest.

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References

1. 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 (3-4) (2012) 807-14.
2. S.G. Wu, Y.N. Liu, M.F. Tsai, Y.L. Chang, C.J. Yu, P.C. Yang, J.C. Yang, Y.F. Wen, J.Y. Shih, The mechanism of acquired resistance to irreversible EGFR tyrosine kinase inhibitor-afatinib in lung adenocarcinoma patients, Oncotarget 7 (11) (2016) 12404-13.
3. M. Campo, D. Gerber, J.F. Gainor, R.S. Heist, J.S. Temel, A.T. Shaw, P. Fidias, A. Muzikansky, J.A. Engelman, L.V. Sequist, Acquired Resistance to First-Line Afatinib and the Challenges of Prearranged Progression Biopsies, J Thorac Oncol 11 (11) (2016) 2022-2026.
4. H.A. Yu, M.E. Arcila, N. Rekhtman, C.S. Sima, M.F. Zakowski, W. Pao, M.G. Kris, V.A. Miller, M. Ladanyi, G.J. Riely, Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers, Clin Cancer Res 19 (8) (2013) 2240-7.
5. T.S. Mok, Y.L. Wu, M.J. Ahn, M.C. Garassino, H.R. Kim, S.S. Ramalingam, F.A. Shepherd, Y. He, H. Akamatsu, W.S. Theelen, C.K. Lee, M. Sebastian, A. Templeton, H. Mann, M. Marotti, S. Ghiorghiu, V.A. Papadimitrakopoulou, A. Investigators, Osimertinib or Platinum-Pemetrexed in EGFR T790M-Positive Lung Cancer, N Engl J Med 376 (7) (2017) 629-640.
6. K.S. Thress, C.P. Paweletz, E. Felip, B.C. Cho, D. Stetson, B. Dougherty, Z. Lai, A. Markovets, A. Vivancos, Y. Kuang, D. Ercan, S.E. Matthews, M. Cantarini, J.C. Barrett, P.A. Janne, G.R. Oxnard, Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M, Nat Med 21 (6) (2015) 560-2.
7. J.C. Soria, Y. Ohe, J. Vansteenkiste, T. Reungwetwattana, B. Chewaskulyong, K.H. Lee, A. Dechaphunkul, F. Imamura, N. Nogami, T. Kurata, I. Okamoto, C. Zhou, B.C. Cho, Y. Cheng, E.K. Cho, P.J. Voon, D. Planchard, W.C. Su, J.E. Gray, S.M. Lee, R. Hodge, M. Marotti, Y. Rukazenkov, S.S. Ramalingam, F. Investigators, Osimertinib in Untreated EGFR-Mutated Advanced Non-Small-Cell Lung Cancer, N Engl J Med 378 (2) (2018) 113-125.
8. Y. Kobayashi, Y. Togashi, Y. Yatabe, H. Mizuuchi, P. Jangchul, C. Kondo, M. Shimoji, K. Sato, K. Suda, K. Tomizawa, T. Takemoto, T. Hida, K. Nishio, T. Mitsudomi, EGFR Exon 18 Mutations in Lung Cancer: Molecular Predictors of Augmented Sensitivity to Afatinib or Neratinib as Compared with First- or Third-Generation TKIs, Clin Cancer Res 21 (23) (2015) 5305-13.
9. K. Uchibori, N. Inase, M. Araki, M. Kamada, S. Sato, Y. Okuno, N. Fujita, R. Katayama, Brigatinib combined with anti-EGFR antibody overcomes osimertinib resistance in EGFR-mutated non-small-cell lung cancer, Nat Commun 8 (2017) 14768.

10. M. Chiba, Y. Togashi, E. Bannno, Y. Kobayashi, Y. Nakamura, H. Hayashi, M. Terashima, M.A. De Velasco, K. Sakai, Y. Fujita, T. Mitsudomi, K. Nishio, Efficacy of irreversible EGFR-TKIs for the uncommon secondary resistant EGFR mutations L747S, D761Y, and T854A, BMC Cancer 17（1）（2017）281.
11. T. Kosaka, Y. Yatabe, H. Endoh, H. Kuwano, T. Takahashi, T. Mitsudomi, Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications, Cancer Res 64（24）（2004）8919-23.
12. M. Bersanelli, R. Minari, P. Bordi, L. Gnetti, C. Bozzetti, A. Squadrilli, C.A. Lagrasta, L. Bottarelli, G. Osipova, E. Capelletto, M. Mor, M. Tiseo, L718Q Mutation as New Mechanism of Acquired Resistance to AZD9291 in EGFR-Mutated NSCLC, J Thorac Oncol 11（10）（2016）e121-3.
13. Y. Liu, Y. Li, Q. Ou, X. Wu, X. Wang, Y.W. Shao, J. Ying, Acquired EGFR L718V mutation mediates resistance to osimertinib in non-small cell lung cancer but retains sensitivity to afatinib, Lung Cancer 118（2018）1-5.
14. A. Oztan, S. Fischer, A.B. Schrock, R.L. Erlich, C.M. Lovly, P.J. Stephens, J.S. Ross, V. Miller, S.M. Ali, S.I. Ou, L.E. Raez, Emergence of EGFR G724S mutation in EGFR-mutant lung adenocarcinoma post progression on osimertinib, Lung Cancer 111（2017）84-87.
15. K. Chen, F. Zhou, W. Shen, T. Jiang, X. Wu, X. Tong, Y.W. Shao, S. Qin, C. Zhou, Novel Mutations on EGFR Leu792 Potentially Correlate to Acquired Resistance to Osimertinib in Advanced NSCLC, J Thorac Oncol 12（6）（2017）e65-e68.
16. S.I. Ou, J. Cui, A.B. Schrock, M.E. Goldberg, V.W. Zhu, L. Albacker, P.J. Stephens, V.A. Miller, S.M. Ali, Emergence of novel and dominant acquired EGFR solvent-front mutations at Gly796（G796S/R）together with C797S/R and L792F/H mutations in one EGFR（L858R/T790M）NSCLC patient who progressed on osimertinib, Lung Cancer 108（2017）228-231.
17. S. Klempner, P. Mehta, A. Schrock, S. Ali, S.-H.I. Ou, Cis-oriented solvent-front EGFR G796S mutation in tissue and ctDNA in a patient progressing on osimertinib: a case report and review of the literature, Lung Cancer: Targets and Therapy Volume 8（2017）241-247.
18. R. Menon, J. Muller, P. Schneider, S. Lakis, K. Thress, J. Wolf, L. Heukamp, J.M. Heuckmann, F. Griesinger, A Novel EGFR（C797）Variant Detected in a Pleural Biopsy Specimen from an Osimertinib-Treated Patient Using a Comprehensive Hybrid Capture-Based Next-Generation Sequencing Assay, J Thorac Oncol 11（9）（2016）e105-7.
19. H.A. Yu, S.K. Tian, A.E. Drilon, L. Borsu, G.J. Riely, M.E. Arcila, M. Ladanyi, Acquired Resistance of EGFR-Mutant Lung Cancer to a T790M-Specific EGFR Inhibitor: Emergence of a Third Mutation（C797S）in the EGFR Tyrosine Kinase Domain, JAMA Oncol 1（7）（2015）982-4.

20. Z. Yang, N. Yang, Q. Ou, Y. Xiang, T. Jiang, X. Wu, H. Bao, X. Tong, X. Wang, Y.W. Shao, Y. Liu, Y. Wang, C. Zhou, Investigating novel resistance mechanisms to third-generation EGFR tyrosine kinase inhibitor osimertinib in non-small cell lung cancer patients, Clin Cancer Res (2018).
21. M. Hidalgo, L.L. Siu, J. Nemunaitis, J. Rizzo, L.A. Hammond, C. Takimoto, S.G. Eckhardt, A. Tolcher, C.D. Britten, L. Denis, K. Ferrante, D.D. Von Hoff, S. Silberman, E.K. Rowinsky, Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies, J Clin Oncol 19 (13) (2001) 3267-79.
22. T.A. Yap, L. Vidal, J. Adam, P. Stephens, J. Spicer, H. Shaw, J. Ang, G. Temple, S. Bell, M. Shahidi, M. Uttenreuther-Fischer, P. Stopfer, A. Futreal, H. Calvert, J.S. de Bono, R. Plummer, Phase I trial of the irreversible EGFR and HER2 kinase inhibitor BIBW 2992 in patients with advanced solid tumors, J Clin Oncol 28 (25) (2010) 3965-72.
23. P.A. Janne, D.S. Boss, D.R. Camidge, C.D. Britten, J.A. Engelman, E.B. Garon, F. Guo, S. Wong, J. Liang, S. Letrent, R. Millham, I. Taylor, S.G. Eckhardt, J.H. Schellens, Phase I dose-escalation study of the pan-HER inhibitor, PF299804, in patients with advanced malignant solid tumors, Clin Cancer Res 17 (5) (2011) 1131-9.
24. P.A. Janne, J.C. Yang, D.W. Kim, D. Planchard, Y. Ohe, S.S. Ramalingam, M.J. Ahn, S.W. Kim, W.C. Su, L. Horn, D. Haggstrom, E. Felip, J.H. Kim, P. Frewer, M. Cantarini, K.H. Brown, P.A. Dickinson, S. Ghiorghiu, M. Ranson, AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer, N Engl J Med 372 (18) (2015) 1689-99.
25. S.N. Gettinger, L.A. Bazhenova, C.J. Langer, R. Salgia, K.A. Gold, R. Rosell, A.T. Shaw, G.J. Weiss, M. Tugnait, N.I. Narasimhan, D.J. Dorer, D. Kerstein, V.M. Rivera, T. Clackson, F.G. Haluska, D.R. Camidge, Activity and safety of brigatinib in ALK -rearranged non-small-cell lung cancer and other malignancies: a single-arm, open-label, phase 1/2 trial, The Lancet Oncology 17 (12) (2016) 1683-1696.
26. Y. Kobayashi, K. Azuma, H. Nagai, Y.H. Kim, Y. Togashi, Y. Sesumi, M. Chiba, M. Shimoji, K. Sato, K. Tomizawa, T. Takemoto, K. Nishio, T. Mitsudomi, Characterization of EGFR T790M, L792F, and C797S Mutations as Mechanisms of Acquired Resistance to Afatinib in Lung Cancer, Mol Cancer Ther 16 (2) (2017) 357-364.
27. D. Ercan, H.G. Choi, C.H. Yun, M. Capelletti, T. Xie, M.J. Eck, N.S. Gray, P.A. Janne, EGFR Mutations and Resistance to Irreversible Pyrimidine-Based EGFR Inhibitors, Clin Cancer Res 21 (17) (2015) 3913-23.
28. J.C. Yang, L.V. Sequist, S.L. Geater, C.M. Tsai, T.S. Mok, M. Schuler, N. Yamamoto, C.J. Yu, S.H. Ou, C. Zhou, D. Massey, V. Zazulina, Y.L. Wu, Clinical activity of afatinib in patients with advanced non-small-cell lung cancer harbouring uncommon EGFR mutations: a combined post-hoc analysis of LUX-Lung 2, LUX-Lung 3, and LUX-Lung 6, The Lancet. Oncology 16 (7) (2015) 830-8.

Supplementary Table 1. List of primers for mutagenesis PCR

	Forward primer	Reverse primer
L718Q	5′-AAAGTGCAGGGCTCCGGTGCGTTC-3′	5′-CACCGGAGCCCTGCACTTTGATCTTTTTGAATTC-3′
L718V	5′-AAAGTGGTGGGCTCCGGTGCGTTC-3′	5′-CACCGGAGCCCACCACTTTGATCTTTTTGAATTC-3′
G724S	5′-GCGTTCAGCACGGTGTATAAGGGACTC-3′	5′-ATACACCGTGCTGAACGCACCGGAG -3′
T790M	5′-CTCATCATGCAGCTCATGCCCTTCGGCTGC-3′	5′-GAGCTGCATGATGAGCTGCACGGTGG-3′
L792F	5′-CACGCAGTTCATGCCCTTCGGCTGCCT-3′	5′-GGCATGAACTGCGTGATGAGTTGCACGGTG -3′
L792H	5′-CACGCAGCACATGCCCTTCGGCTGCCT-3′	5′-GGCATGTGCTGCGTGATGAGTTGCACGGTG -3′
G796S	5′-CCCTTCAGCTGCCTCCTGGACTATG-3′	5′-GAGGCAGCTGAAGGGCATGAGCTGCG-3′
C797S	5′-TTCGGCAGCCTCCTGGACTATG-3′	5′-AGGAGGCTGCCGAAGGGCATGAGCTGCGT-3′
C797G	5′-TTCGGCGGCCTCCTGGACTATG-3′	5′-AGGAGGCCGCCGAAGGGCATGAGCTGCGT-3′
G796S *	5′-TGCCCTTCAGCTGCCTCCTGGACTATG-3′	5′-GGCAGCTGAAGGGCATGAGCTGCATGATGAG-3′
C797G *	5′-CCTTCGGCGGCCTCCTGGACTATGTCCGG-3′	5′-GGAGGCCGCCGAAGGGCATGAGCTG-3′
C797S *	5′-CCTTCGGCAGCCTCCTGGACTATGTCCGG-3′	5′-GGAGGCTGCCGAAGGGCATGAGCTG-3′
L858R	5′-GCGGGCCAAACTGCTGGGTGC-3′	5′-AGCAGTTTGGCCCGCCCAAAATCTGTGATCTTG-3′

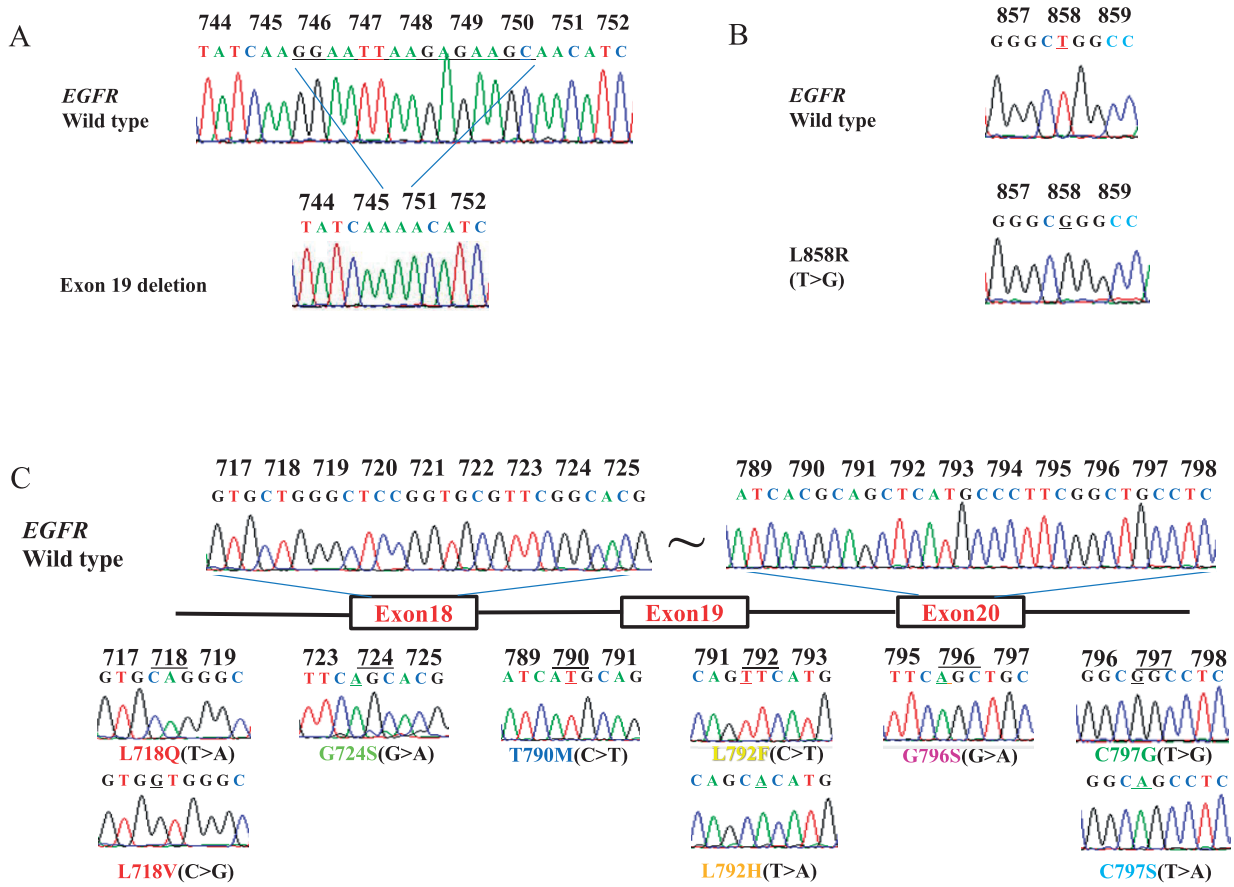
* Primers used to induce each mutation after T790M induction.

Supplementary Table 2. IC_{50} value of each transfected Ba/F3 cells (exon 19 deletion) against each TKI

	IC_{50} (nM)				
EGFR mutation	Erlotinib	Afatinib	Dacomitinib	Osimertinib	Brigatinib
Del19	1.20	0.047	0.12	0.91	17.64
Del19+L718V	14.79	0.464	0.295	10.57	322.12
Del19+G724S	1.93	0.025	0.025	3.79	55.71
Del19+T790M	6685.99	143.95	270.66	0.70	289.85
Del19+L792F	3.31	0.30	0.19	4.01	155.08
Del19+L792H	11.61	0.57	0.073	11.95	140.74
Del19+C797S	3.33	4.78	5.88	925.37	70.24

Supplementary Table 3. IC_{50} value of each transfected Ba/F3 cells (L858R) against each TKI

	IC_{50} (nM)				
EGFR mutation	Erlotinib	Afatinib	Dacomitinib	Osimertinib	Brigatinib
L858R	9.37	0.616	0.12	2.34	277.24
L858R+L718Q	424.56	3.37	1.99	533.04	629.40
L858R+L718V	184.93	0.703	0.229	167.90	793.86
L858R+T790M	5596.16	99.52	94.177	2.80	335.11
L858R+L792F	14.87	0.97	0.21	29.53	556.81
L858R+L792H	28.15	1.25	0.16	44.71	662.25
L858R+C797G	1.26	1.38	2.18	561.21	58.31
L858R+C797S	4.81	6.68	4.63	917.95	174.28



Supplementary Figure 1. Direct sequencing of activating EGFR mutations and osimertinib-resistance point mutations introduced into Ba/F3 cells. (A) Activating mutations Del19 and L858R. (B) Each osimertinib-resistance point mutation.