Identification and characterization of preexisting resistant subclone in lung cancer with *EGFR* mutation sensitive to EGFR tyrosine kinase inhibitor

Youngjoo Lee

Department of Medicine

The Graduate School, Yonsei University

Identification and characterization of preexisting resistant subclone in lung cancer with *EGFR* mutation sensitive to EGFR tyrosine kinase inhibitor

Directed by Professor Joon Chang

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Youngjoo Lee

December 2015

This certifies that the Doctoral Dissertation of Youngjoo Lee is approved.

Thesis Supervisor : Joon Chang

Thesis Committee Member#1 : Joo-Hang Kim

Thesis Committee Member#2 : Se Kyu Kim

Thesis Committee Member#3 : Jae J. Song

Thesis Committee Member#4 : Jaeheon Jeong

The Graduate School
Yonsei University

December 2015

ACKNOWLEDGEMENTS

I would like to express my special appreciation and thanks to my advisors Professor Dr. Kim and Professor Dr. Chang, you have been tremendous mentors for me. I would like to thank you for encouraging my research and for allowing me to grow as a medical scientist. Your advice on the research as well as on my career has been priceless. I would also like to thank my committee members, Professor Se Kyu Kim, Professor Jae J. Song, Professor Jaeheon Jeong for serving as my committee members even at hardship. I also want to thank you for letting my defense be an enjoyable moment, and for your brilliant comments and suggestions, thanks to you. I would especially like to thank Dr. Jin Soo Lee, Dr. Heung Tae Kim, Dr. Ji-Youn Han, and Dr. Geon Kook Lee aids in Center for Lung Cancer at National Cancer Center. All of you have been there to support me when I recruited patients and collected data and tissue samples for my Ph.D. thesis. I also appreciate all patients and their families who agreed with the use of their tissues for my research.

A special thanks to my family. Words cannot express how grateful I am to my mother, father, mother-in law, and father-in-law, for all of the sacrifices that you've made on my behalf. Your support on me was what sustained me thus far. I would also like to thank all of my friends who supported me

in writing, and incented me to strive towards my goal. At the end I would like express appreciation to my beloved husband Sung Wook and beautiful daughter Geena who were always my support in every moment.

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

Identification and characterization of preexisting resistant subclone in lung cancer with *EGFR* mutation sensitive to EGFR tyrosine kinase inhibitor

Youngjoo Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Joon Chang)

EGFR tyrosine kinase inhibitor (EGFR-TKI) is the first molecularly targeted drug to change the paradigm in the management of lung cancer. These drugs are specifically effective in lung cancers with activating *EGFR* mutations. However, most cancer that initially responds to EGFR-TKI eventually acquires drug resistance. Several mechanisms are responsible for acquired resistance to EGFR-TKI, and the most common is the emergence of the T790M mutation in EGFR. This study aimed to evaluate whether the tumor cells carrying the T790M mutation exists before drug exposure and expands under the selective drug pressure. We collected pretreatment tumor tissues from 124 advanced non-small cell lung cancer patients with activating *EGFR* mutations that were detected by direct sequencing. Genotyping for T790M by matrix-assisted laser desorption/ionization-time of flight/mass spectrometry identified 31 (25.0%) tumors with pretreatment T790M. Furthermore, 68 cases which additionally underwent droplet digital PCR showed 27 (39.7%) tumors had pretreatment T790M mutation. We also observed clonal expansion of preexisting T790M clones during EGFR-TKI treatment both in in vivo model and in the paired tissue samples. In co-culture study mixing

drug-sensitive cell and luciferase-tagging drug-resistant cell, we showed this growth advantage of resistant cell in regressing tumors was caused by dying sensitive cells. The T790M mutation frequency at which the risk of progression to EGFR-TKI begins to increase was estimated to be 3.2%. The patients with T790M-positive tumor had shorter time to progression (TTP) after EGFR-TKI (median 6.3 months vs. 11.5 months; $P < 0.001$) and overall survival (OS) (median 16.1 months vs. 26.5 months; $P = 0.065$) than those with T790M-negative tumor. Among the T790M-positive patients, the patients with high T790M frequency (n= 9) had shorter TTP (median 2.4 months vs. 6.7 months; $P = 0.009$) and OS (median 9.1 months vs. 18.7 months; $P = 0.018$) than those with low T790M frequency (n= 22). In conclusions, EGFR T790M mutation preexisted substantially in EGFR-mutant lung cancer. The T790M clones may expand during EGFR-TKI treatment by dying sensitive cell and finally be responsible for the patients' resistance and progression. Thus, the patients with high T790M mutation frequency had worse clinical outcomes to EGFR-TKI.

Keywords: Lung cancer, targeted therapy, EGFR, tyrosine kinase inhibitor, acquired resistance, EGFR T790M

Identification and characterization of preexisting resistant subclone in lung cancer with EGFR mutation sensitive to EGFR tyrosine kinase inhibitor

Youngjoo Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Joon Chang)

I. INTRODUCTION

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) have shown dramatic response in patients with non-small cell lung cancer (NSCLC) harboring sensitizing *EGFR* mutations.[1] However, the majority of the patients with *EGFR*-mutant lung cancer who initially respond to EGFR-TKI therapy eventually acquire resistance to the drug and experience disease progression. The T790M gatekeeper mutation in the *EGFR* gene is regarded as the most common cause of acquired resistance to EGFR-TKI.[2, 3] This mutation causes substitution of threonine to methionine at codon 790 in the catalytic domain of EGFR. This substitution increases the receptor's affinity for adenosine triphosphate and impairs receptor-drug binding by inhibiting hydrogen bond formation or inducing steric hindrance.[2, 4] This T790M resistant

mutation was found in approximately 60% of rebiopsy samples obtained from patients with acquired resistance to EGFR-TKI therapy.[5]

A subset of patients with sensitive *EGFR* mutations did not respond to EGFR-TKI therapy or their response was shorter than expected.[6-8] The cause of this reduction in the efficacy of targeted therapy in a molecularly defined patient population remains unknown. Recently, some case reports and preclinical studies have suggested that the T790M resistance mutation may exist before EGFR-TKI exposure and may play a fundamental role in the inherent resistance of *EGFR*-mutant tumors to EGFR-TKI therapy.[9, 10] Based on these findings, it was assumed that some subclones with this resistant mutation are present before starting EGFR-TKI therapy, albeit at a low frequency, and selective pressure from EGFR-TKI therapy may thus cause the expansion of these latent resistant subclones, resulting in the emergence of a resistant phenotype in the entire population of cells.

Several studies have tried to prove the presence of preexisting T790M mutation in *EGFR*-mutant lung cancer using genotyping methods that are more sensitive than the commonly used direct sequencing method, which is usually unable to detect low-frequency mutations.[11-16] However, the frequency of preexisting T790M mutations reported in prior studies was

controversial. Additionally, there was no data about the proportion of these resistant clones within each tumor or the clinical outcomes according to the proportion of these clones. Especially, it is unclear whether the acquisition of whole-tumor resistance is driven by resistant tumor cells that are present at a low frequency before starting therapy. Thus, we try to identify and characterize latent resistant subclone with T790M mutation in lung cancer with *EGFR* mutation sensitive to EGFR tyrosine kinase inhibitor.

II. MATERIALS AND METHODS

Patients and tumor samples

Advanced NSCLC patients who underwent routine direct sequencing of the *EGFR* gene and who were treated with an EGFR-TKI (e.g., gefitinib or erlotinib) at the National Cancer Center Hospital (Goyang, Republic of Korea) between January 2009 and August 2014 were screened for this study (Figure 1). Archival formalin-fixed, paraffin-embedded pre-treatment or posttreatment tumor tissues were collected for genetic analysis. A pathologist (K.G.L) reviewed hematoxylin-eosin stained sections of each tissue sample and identified tissue areas containing ≥70% tumor for dissection. Genomic DNA was extracted from the dissected tumor tissue using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

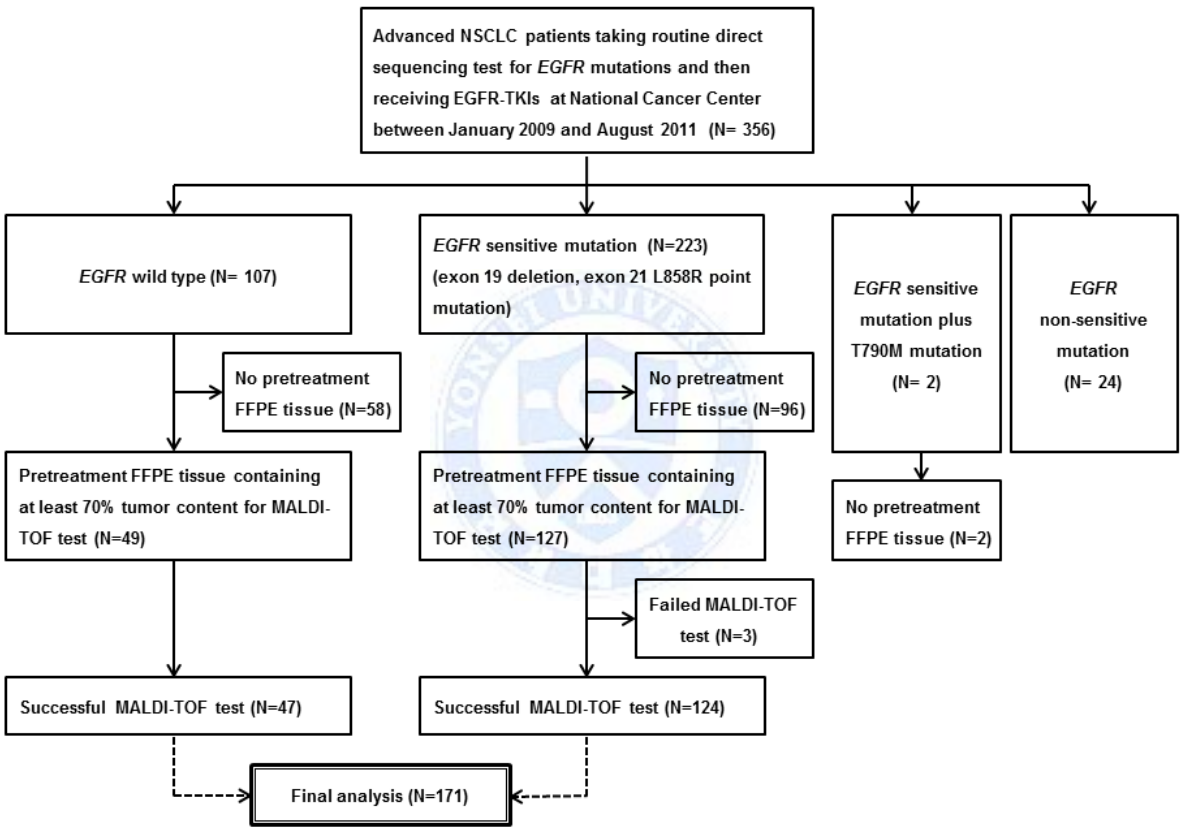

Figure 1. Patient selection and tissue collection.

Direct sequencing analysis

Polymerase chain reaction (PCR) amplification was performed with 5 μl of the extracted genomic DNA, 1 U of *Taq* DNA polymerase, 0.25 mM each dNTP, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, and 20 pmol of the primers in a final volume of 20 μl. The following primers were used to amplify exon 20 of *EGFR*: 5′-CCATGAGTACGTATTTTG-AAACTC-3′ (forward) and 5′-CATATCCCCATGGCAAACTCTTGC-3′ (reverse). The PCR cycling parameters were 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final step at 72 °C for 10 min. After the PCR products were purified, they were directly sequenced with the MegaBACE DNA Analysis System (Amersham Biosciences, Sunnyvale, CA, USA), with a standard published protocol.

Cases harboring the sensitive mutations (exon 19 deletion and exon 21 L858R) except those with coexisting T790M mutations were further genotyped for the T790M mutation in exon 20. In order to more sensitively genotype the T790M mutation, two genotyping methods including matrix-assisted laser desorption/ ionization-time of flight/MS (MALDI-TOF/MS) (Sequenom, San Diego, CA) and droplet digital polymerase chain reaction (ddPCR) (Bio-Rad, Hercules, CA, USA) were

used.

Sensitivity analysis of the MS assay

Serial DNA mixtures of mutant (H1975) and wild-type (HCC827) DNA were genotyped by the MS assay to determine the detection limit and cutoff value for the T790M mutation (Figure 2A, B). The T790M mutant signal calls were detectable in the cluster plot and mass spectrum of the DNA mixtures with a T790M mutation frequency of ≥2.5%. The mutant signal frequency was calculated as follows: mutant signal frequency (%) = (mutant peak height) / (mutant peak height + wild-type peak height) × 100. The calculated mutant signal frequency in the 2.5% T790M DNA mixture was 2.95%. Thus, this value was used as the cutoff value for the T790M mutation in further analysis. The diluted mutant DNA percentage was linearly correlated with the mutant signal frequency (R^2 = 0.9878) (Figure 2C).

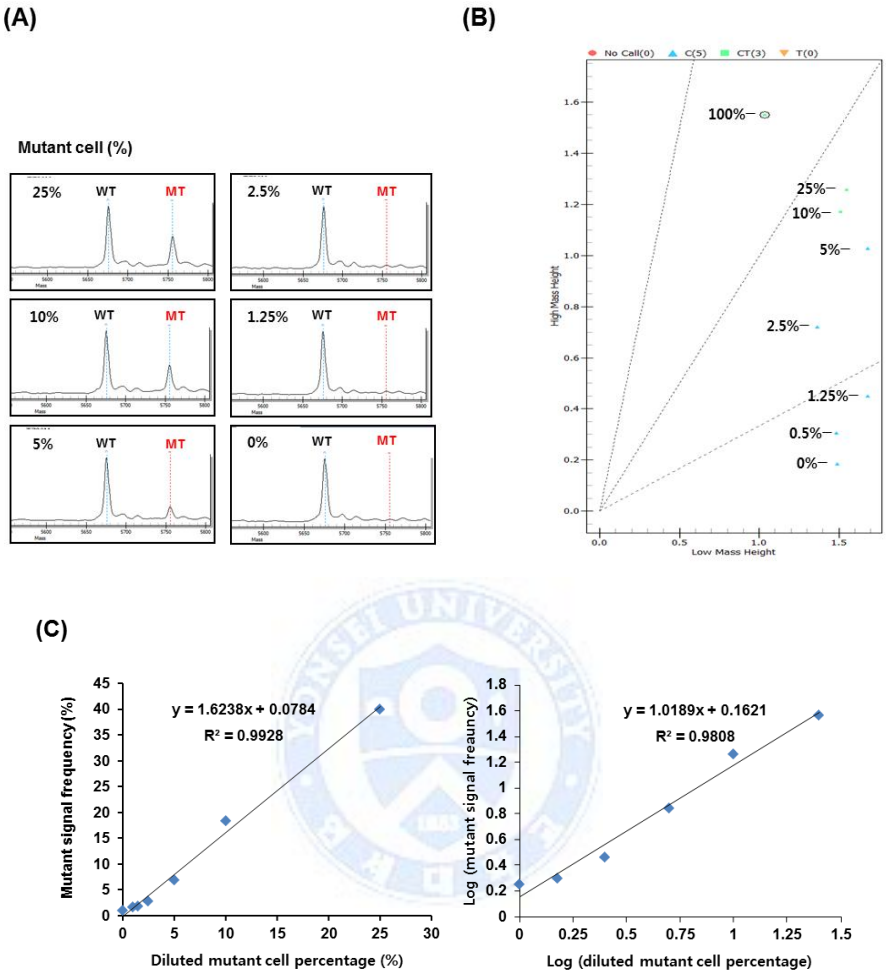

Figure 2. Determination of the detection limit for the T790M mutation (790C>T, exon 20) by MALDI-TOF/MS using a serial DNA mixture of mutant and wild-type DNA. Mutant and wild-type DNA was extracted from H1975 cells and H827 cells, respectively, and was mixed at the indicated proportions. In the mass spectrum (A) and call cluster plot (B), a peak corresponding to the T790M mutant can be seen in the diluted mixtures containing ≥2.5% of mutant DNA. (C) The diluted mutant DNA percentage was linearly correlated with the mutant signal frequency in the genotyping assay. WT: wild-type peak, M: mutant peak.

Estimating the allele frequency of T790M in cell lines

The allele frequency of EGFR T790M after gefitinib (LC Laboratories, Woburn, MA) or cisplatin treatment was measured in two pairs of cell line mixtures containing TKI-sensitive cells and TKI-resistant cells with the T790M mutation: HCC827/H1975 and PC-9/PC-9GR-T790M[20]. The HCC827, PC-9, and H1975 cell lines were purchased from Korean Cell Line Bank (Seoul, Korea), RIKEN BioResource Center Cell Bank (Ibaraki, Japan), and American Type Culture Collection (Manassas, VA), respectively. TKI-sensitive cells and TKI-resistant cells with the T790M mutation were mixed at the indicated proportions and were treated with gefitinib or cisplatin for 72 hours. And then the ddPCR was performed for measuring of T790M allele frequency.

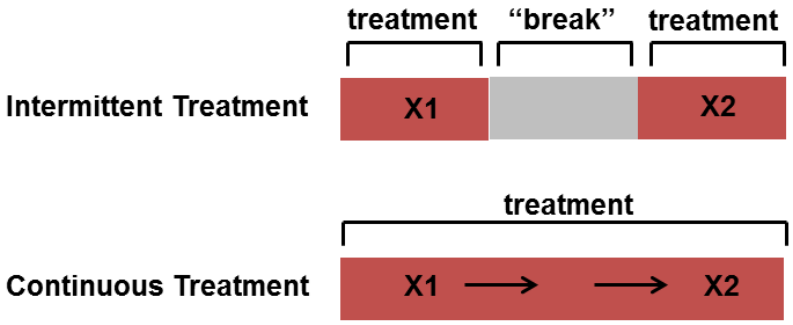

Figure 3. Schematic summary of two drug treatment methods

Establishment of gefitinib-resistant cell lines

The gefitinib-resistant cell line (PC-9GR) was generated by continuously exposing PC-9 cells to increasing concentrations of gefitinib. Starting at a concentration of 0.01 μM, the exposure dose was doubled until it reached a final concentration of 1.0 μM. We used two different drug treatment regimens, intermittent and continuous exposure (Figure 3). Cells in the intermittent treatment group (PC-9/GRi) were exposed to gefitinib in culture medium for 72 h, washed, and then cultured in gefitinib-free medium until their growth rate was similar to that of the parental cells. The cells in the continuous treatment group (PC-9/GRc) were continuously exposed to gefitinib at a given concentration and the medium was not changed to drug-free medium at any time. When the

growth rate of these cells was equal to that of the parental cells, they were exposed to increasing concentrations of gefitinib. The drug-resistant phenotypes of both groups of cells were confirmed with a cell viability assay. And the MS assay was performed for measuring of T790M allele frequency.

Luciferase gene transduction into PC9GR-T790M

We used the pLEX system, a lentivector system purchased from Open Biosystems (Huntsville, AL). The firefly luciferase gene (Fluc) obtained commercially from Promega (Madison, Wisconsin) were cloned into this vector. All the lentiviral vectors were packaged into live lentiviral viruses in 293T cells following manufacturer's instructions.

Bioluminescence imaging

For imaging luciferase, we used the IVIS200 instrument from Caliper Life Sciences (Hopkinton, MA). For tissue cultured cells, we imaged luciferase signal by adding PBS or colorless OptiMEM medium (Invitrogen, Carlsbad, CA) with D-luciferin (Caliper Life Sciences, Nature Medicine doi:10.1038/nm.2385 iv Hopkinton, MA) at a concentration of 0.15 mg/ml. It was important to image the cells at a set

time point (e.g. 10 minutes) after the administration of D-luciferin so that signals from different samples were comparable. After images were taken, we used manufacturer supplied software to process the images for quantitative data.

To monitor growth of luciferase-tagging cells *in vitro*, 500 luciferase-tagging PC-9GR-T790M cells were mixed together with 5000 unlabeled PC-9 cells, cultured for 5 days, and measured by use of IVIS200.

Growth of luciferase-tagging cells *in vivo* was followed through non-invasive bioluminescence imaging using the IVIS200 instrument (Caliper Life Sciences, Hopkinton, MA). Mice to be imaged were injected with 150 mg/kg of D-luciferin (obtained from Caliper Life Sciences) intraperitoneally in 200μl of PBS and then anesthetized with continuous flow of isofluorane. Imaging of the mice was carried out 10 minutes later.

Monitoring luciferase-tagging tumor cell in mice

The tumor cell mixtures of PC-9 and luciferase-tagging PC-9GR-T790M were subcutaneously injected into the nomobese diabetic/ severe combined immunodeficient (NOD/SCID) mice. Groups of mice were inoculated with each cell line at 5 x 10^6. Individual tumor volumes were

measured using a digital caliper and about according to the formular $V= 1/2ab^2$ (a, the long diameter and b, the short diameter of the tumor). When tumor volume was more than 100 m^3, 5mg/kg of gefitinib was administrated via mouth daily for 5 days/week. Tumor growth from luciferase-tagging PC-9GR-790M is monitored through the quantification of bioluminescence signals emitted from labeled tumor cells by use of the IVIS200 instrument following manufacturer's instructions.

Cell viability assay

The cells were then seeded at a density of 4×10^3 cells/well in 96-well plates. After 24 h, the cells were exposed to different concentrations of gefitinib and were incubated for 72 h. The cells were then washed with phosphate-buffered saline and the cell viability was measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Statistical analysis

Differences between pairs of categorical variables were analyzed using the χ^2 test or Fisher's exact test. Time to progression (TTP) was measured

from the first day of starting EGFR-TKI until the identification of disease progression (PD). Overall survival (OS) was calculated from the first day of starting EGFR-TKI until death or the most recent follow-up. Survival time was estimated using the Kaplan–Meier method and was compared between groups using the log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate survival analyses to determine hazards ratios (HR) with 95% confidence intervals (CI). Two-sided values of $P < 0.05$ were considered statistically significant.

III. RESULTS

Identification of preexisting EGFR T790M mutations in EGFR-*mutant lung cancer*

T790M was analyzed by direct sequencing, MALDI-TOF MS, and ddPCR methods in EGFR-TKI-treated patient cohorts with advanced NSCLC. In the pretreatment tissue, direct sequencing could not identify any patients with T790M, whereas the MS assay detected 31 of 124 (25.0%). Among them, 68 with adequate DNA amount and quality were further examined by the ddPCR assay. The detection sensitivity of T790M mutation by the ddPCR assay was higher than that of the MS assay (27 of 68, 39.7%) (Figure 4A). We calculated the ratio of the number of T790M alleles to that of activating mutant alleles (T/A) to show the distribution of latent T790M burden in this study group (Figure 4B). The mean value of T/A ratio was 0.5% (range, 0.0%-7.3%) with 1.12 of standard deviation.

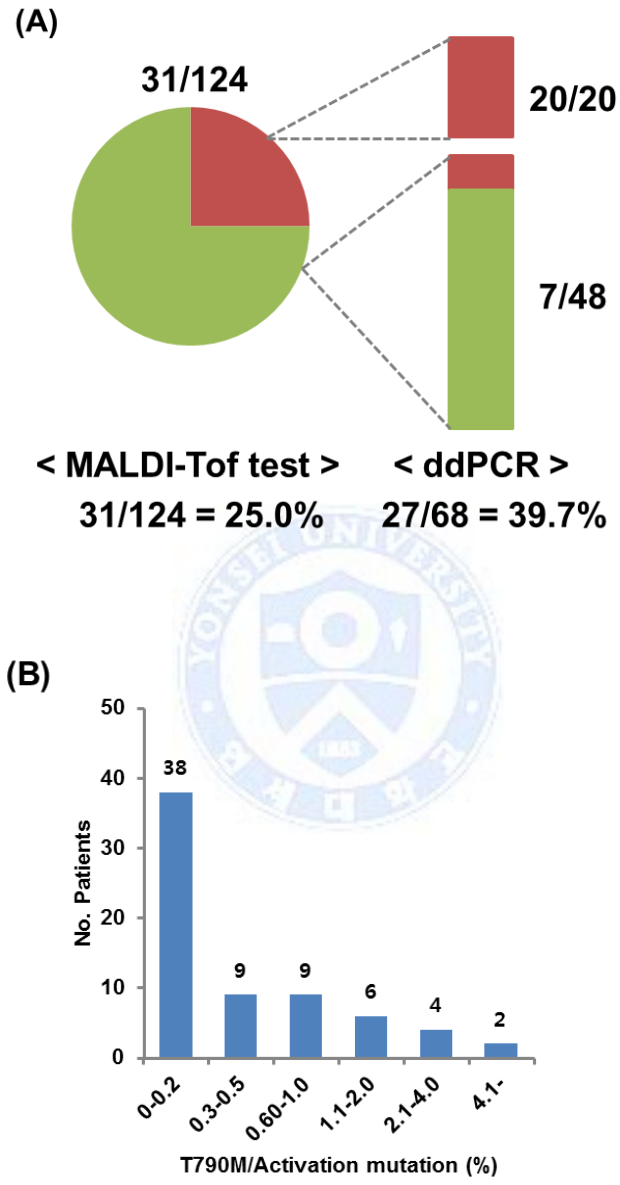

Figure 4. (A) The frequency of patients carrying pretreatment *EGFR* T790M. (B) Distribution of intratumoral T790M/Activating mutation allele frequency ratio in 27 pretreatment *EGFR*-mutant lung cancer tumors.

Clonal expansion of preexisting EGFR T790M clones during EGFR-TKI treatment

The T790M/activating mutation allele frequency ratio after gefitinib or cytotoxic chemotherapy was calculated in two pairs of cell line mixtures containing TKI-sensitive cells and TKI-resistant cells with the T790M mutation (HCC827/H1975 and PC-9/PC-9GR-T790M). In Figure 5, TKI-resistant cells carrying the T790M mutation (H1975 and PC-9GR-T790M) were spiked into TKI-sensitive cells (HCC827 and PC-9) at various proportions (0%, 1%, 5%, and 25%). We found that gefitinib selected significantly better resistant cells compared with control and cisplatin treatment groups.

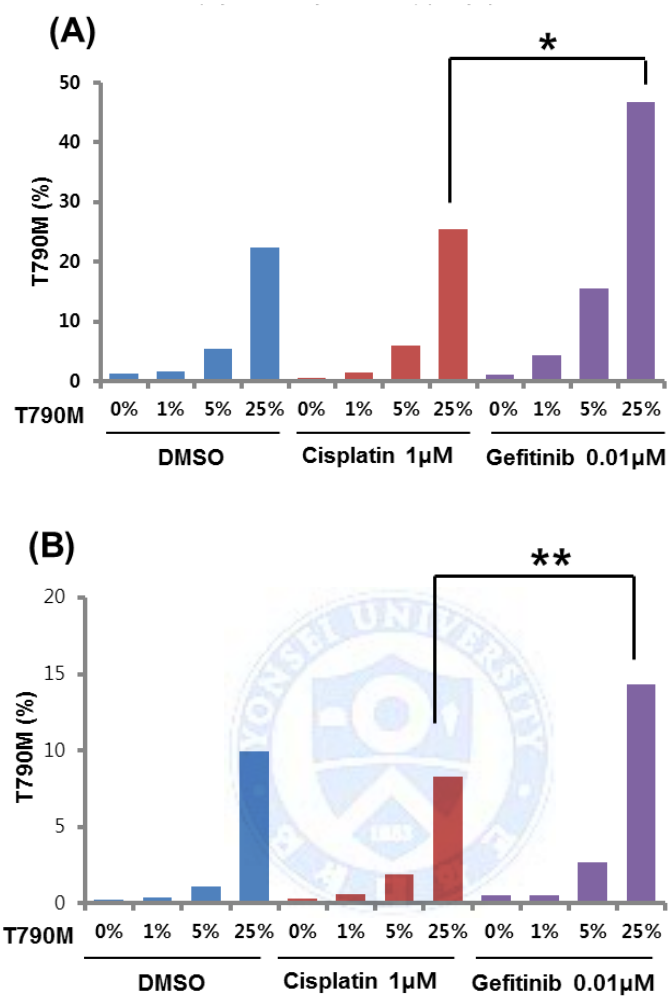

Figure 5. The T790M/activating mutation allele frequency ratio after gefitinib or cisplatin treatment in the mixture of lung cancer cell lines. (A) HCC827 + H1975 (%). (B) PC9 + PC9-GR-T790M (%).The ddPCR assay was done. *P= 0.001, **P= 0.005

To model heterogeneous tumor cell population *in vivo*, we mixed a small percentage of PC-9GR-T790M cells, labelled with luciferase vector, together with mostly non-labelled, PC9 cells, and injected the admixture (PC9/PC9GR, 98%/2% or 75%/25%) subcutaneously in mice. After the tumors were established, we treated the mice with gefitnib and monitored the growth of PC-9GR-T790M resistant cells by bioluminescene imaging in vivo. Although gefitinib treatment decreased the volume of the tumors, the number of admixed resistant cells in regressing tumors increased (Figure 6).

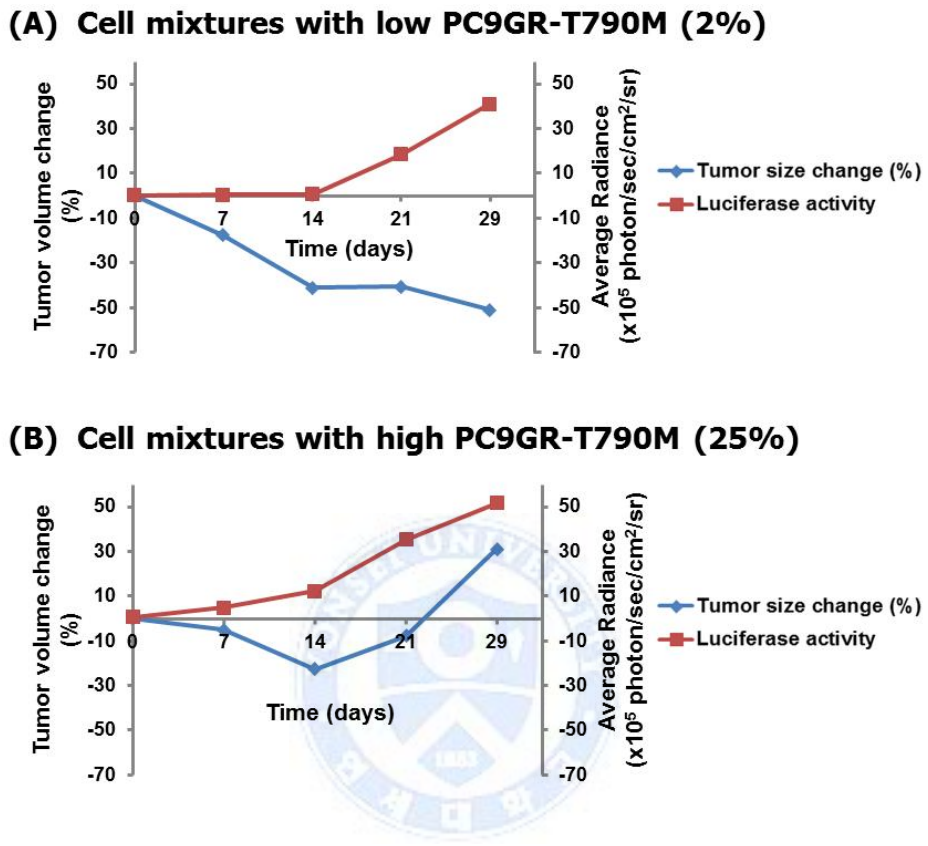

Figure 6. Expansion of luciferase-tagging PC-9GR-T790M cells during gefitinib treatment in xenograft mouse model. The transplanted cell mixtures included (A) low PC-9GR-T790M (2%) and (B) high PC-9GR-T790M (25%).

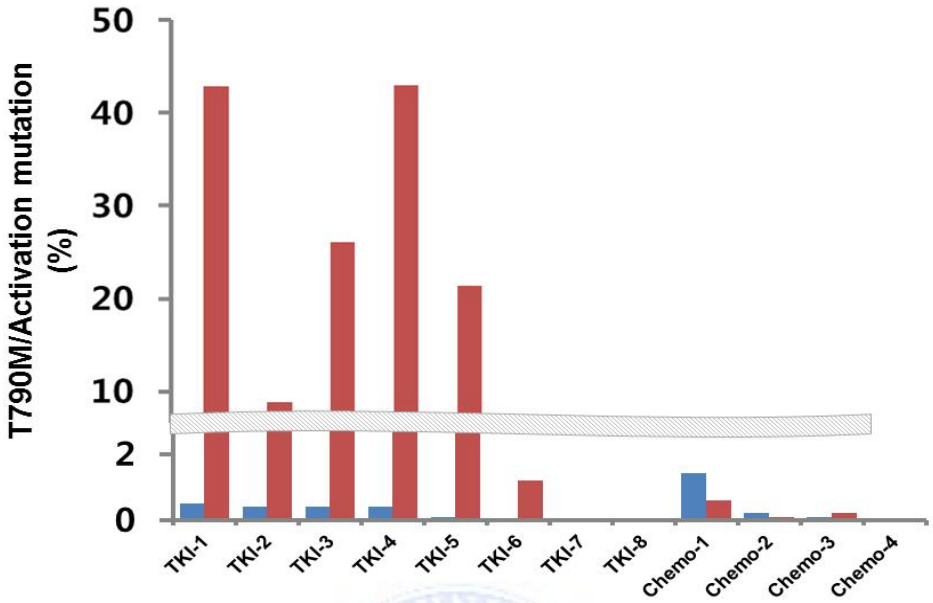

Figure 7. Change in the T790M/activating mutation allele frequency ratio after EGFR-TKI or cytotoxic chemotherapy. The ddPCR assay was done.

We indirectly confirmed these experiment study findings by using the patient samples. Using the ddPCR assay, we measured the T/A ratio of the T790M in 12 paired tumor tissues, which were sampled before and after EGFR-TKI or cytotoxic chemotherapy. As shown in Figure 7, the EGFR-TKI treatment group demonstrated that the presence of T790M before treatment was correlated to the occurrence of T790M after treatment and the percentage of posttreatment T790M increased as that of pretreatment T790M increased. However, the chemotherapy treatment group showed the T790M percentage was almost not changed after

treatment. These results suggested the clonal expansion of preexisting latent T790M cell only under EGFR-TKI treatment.

Growth stimulation from sensitive cell dying under drug treatment

To evaluate whether the growth advantage of resistant cell in regressing tumors was caused by dying sensitive cells, we performed in vitro experiments including coculture assay and indirect culture assay using cell supernatants. Drug-resistant cells (PC-9GR-T790M) tagged with luciferase were cultured together with drug-sensitive cell (PC-9) and then their luciferase activity was measured. At first, we confirmed the linear correlation between measured activity and actual cell numbers (Figure 8A). Based on the measured luciferase activity, we observed that drug-sensitive cell suppressed the proliferation of drug-resistant cell but dying drug-sensitive cell under EGFR-TKI promoted the regrowth of drug-resistant cell (Figure 8B).

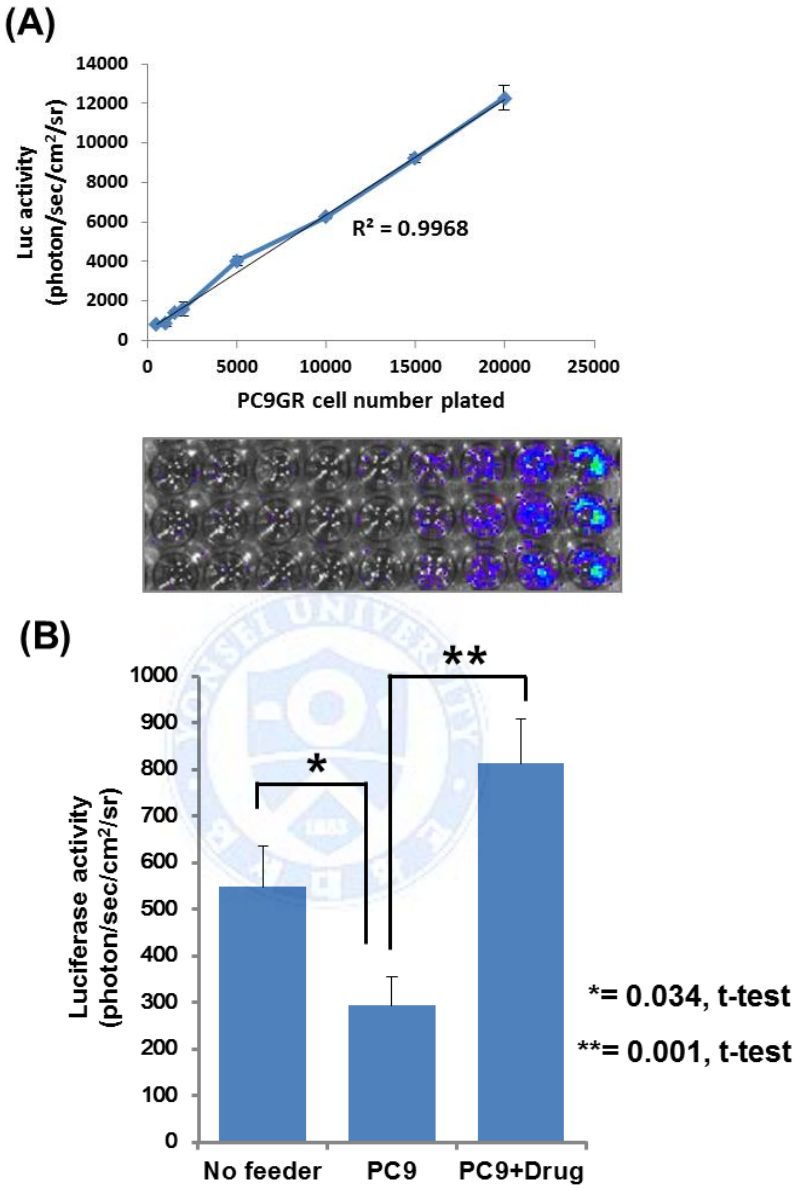

Figure 8. (A) Correlation between measured luciferase activity and cell numbers of luciferase-tagging PC-9GR-T790M. (B) Difference in luciferase activity of luciferase-tagging PC-9GR-T790M according to conditions of 5- day coculture. Drug: gefitinib= 0.01 μM.

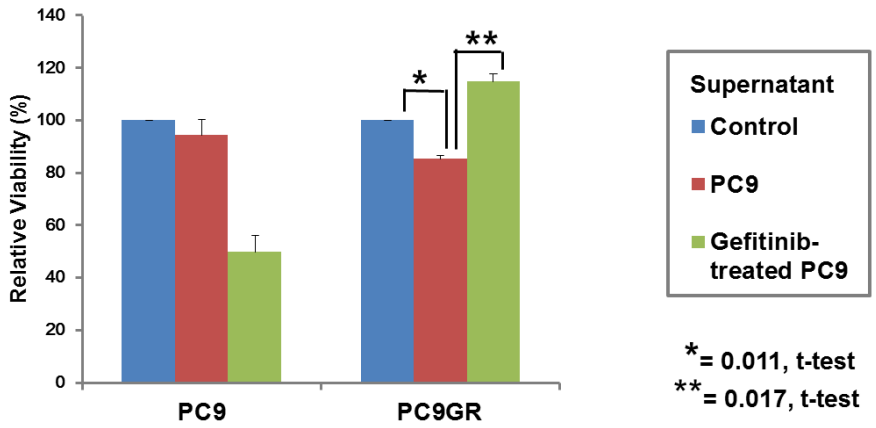

Figure 9. Difference in cell viability of PC-9GR-T790M in indirect study. PC-9 and PC-9GR-T790M cells incubated for 72h in three conditioned media and the cell viability assay was done in each group.

We derived conditioned media (CM) from gefitinib-sensitive cells cultured in the absence (CM-vehicle) or presence of gefitinib (CM-gefitiib). CM-vehicle suppressed the growth of gefitinib-resistant cells whereas CM-gefitinib significantly accelerated, as determined by cell viability assays and bioluminescene activity measurement (Figure 9). Because all biologically active CM was collected before cell death or senescence, it is likely that the secretome is actively produced as a result of oncogene inhibition. These results demonstrate that *EGFR* mutant cells respond to therapeutic stress under targeted therapy by secreting factors that accelerate the growth of drug resistant minority clones.

Effects of the T790M mutation on EGFR-TKI efficacy

The response rate (RR) and disease control rate (DCR) were not significantly different between patients with T790M-positive mutant tumors and T790M-negative mutant tumors (RR: 71.0% vs. 83.9%, P = 0.115; DCR: 83.9% vs. 92.5%, P = 0.173). However, the median TTP was significantly shorter in patients with T790M-positive mutant tumors than in patients with T790M-negative mutant tumors (6.3 months vs. 11.5 months; $P < 0.001$) (Figure 10A). Multivariate analysis of TTP showed that the risk of progression was significantly higher in patients with T790M-positive mutant tumors than in patients with T790M-negative mutant tumors (HR 2.43, 95% CI 1.55–3.80, $P < 0.001$). The shorter TTP of T790M-positive mutant tumors was also observed at the 1st line EGFR-TKI subgroup (6.0 months vs. 10.5 months; HR 2.06, 95% CI, 0.94–4.48; P = 0.062) and at the second-line EGFR-TKI or later subgroup (6.3 months vs. 11.5 months; HR 2.65, 95% CI, 1.53–4.60; P = 0.001) (Figure 10B,C).

The median OS was shorter in T790M-positive mutant group than in T790M-negative mutant group (16.1 months vs. 26.5 months; HR 2.00, 95% CI, 1.14–3.53; P = 0.065) (Figure 10D). However, there was no significant difference in the median survival after the failure of second

EGFR-TKI between two groups (7.9 months vs. 14.2 months; HR 1.59, 95% CI, 0.90-2.78; P = 0.383). These trends remained unchanged according to the presence or absence of exposure to chemotherapy drugs before EGFR-TKI therapy.

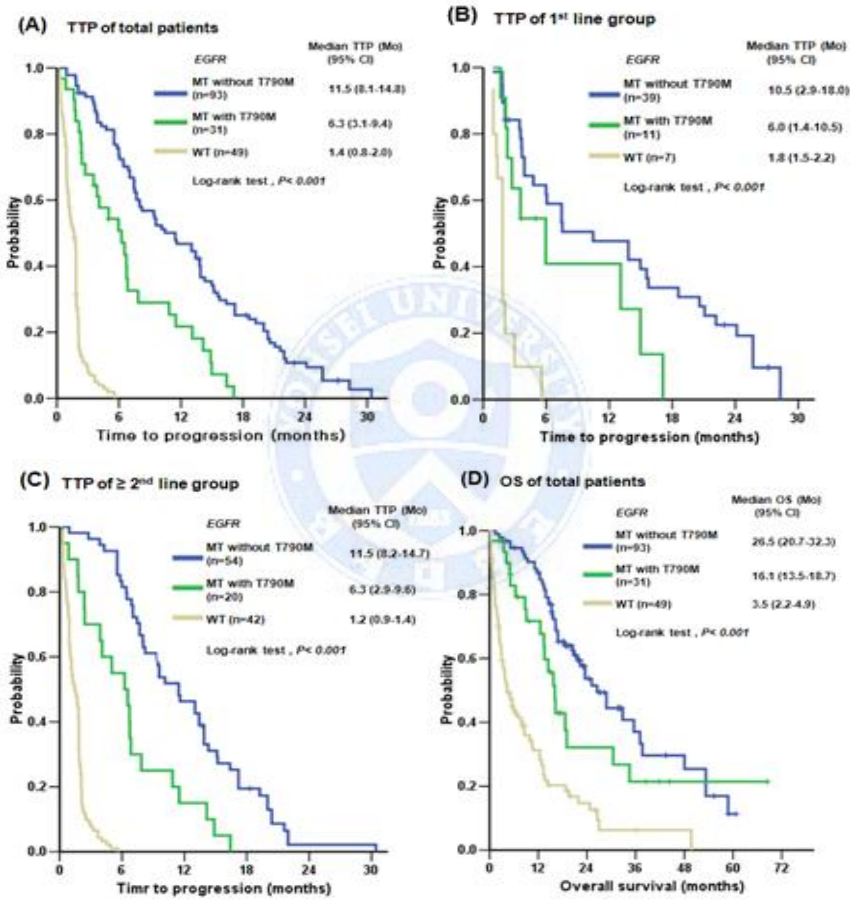

Figure 10. Kaplan–Meier survival curves. (A) TTP of total patients, (B) TTP of 1st line group, (C) TTP of ≥ 2nd line group, and (D) OS of total patients in EGFR wild-type and EGFR-mutant patients divided into two subgroups according to the presence of preexisting T790M mutations.

Dose-dependent effect of the T790M mutation

The optimal cut-off point of T790M mutant signal frequency which gives the most significant HR of progression to EGFR-TKI in T790M-positive mutant tumors was 45.7%, which corresponded to percentages of cells within a tumor of 28.1%, based on the cell-line mixture study. Patients with T790M-positive mutant tumors were divided into two subgroups according to this cut-off value of T790M mutant signal frequency. There was no significant difference in RR and DCR between high (n= 9) and low T790M groups (n= 22) (RR: 66.7% vs. 72.7%, P = 1.000; DCR: 66.7% vs. 90.9%, P = 0.131). However, the median TTP was significantly shorter in high T790M patients than in low T790M patients (2.4 months vs. 6.7 months; HR 2.91, 95% CI, 1.26–6.75; P = 0.009) (Figure 10E). Additionally, the median OS was significantly shorter in high T790M patients than in low T790M patients (9.1 months vs. 18.7 months; HR 6.14, 95% CI, 1.66–22.77; P = 0.018) (Figure 10H). However, there was no significant difference in the median post-TKI survival between high T790M patients and low T790M patients (7.9 months vs. 8.4 months; HR 2.08, 95% CI, 0.81-5.33; P = 0.122).

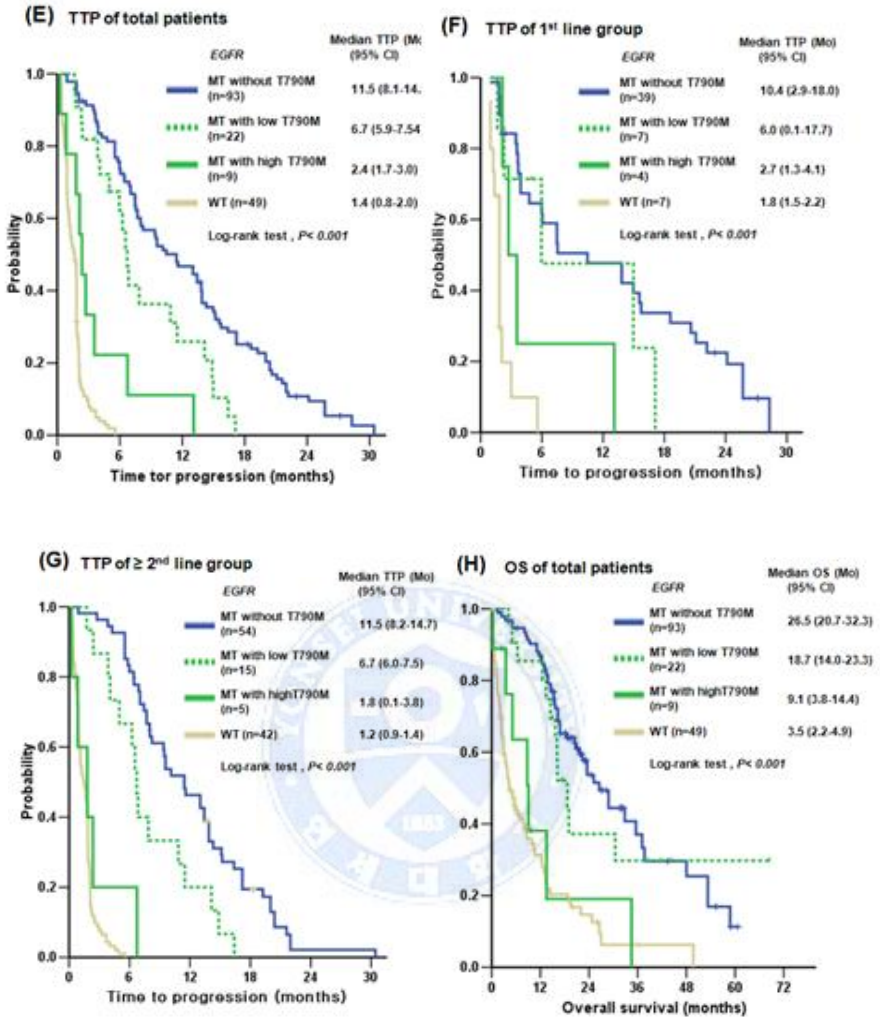

Figure 10. Kaplan–Meier survival curves (E) TTP of total patients, (F) TTP of 1st line group, (G) TTP of ≥ 2nd line group, and (H) OS of total patients in EGFR wild-type and EGFR-mutant patients group, including T790M-negative patients and T790M-postive patients who were divided two subgroups according to the T790M mutant signal frequency.

Estimated frequency of T790M mutant cells for inducing resistance

A total of 124 *EGFR*-mutant patients were divided into two groups by the cutoff level defined as the median value of deciles of the T790M mutant signal frequency. The trends in HRs for TTP during EGFR-TKI therapy according to the cutoff levels of the T790M signal frequency. The minimum cutoff level of the T790M signal at which the risk of progression significantly increased during EGFR-TKI therapy was 5.3%, which corresponds to 3.2% of the estimated percentage (Figure 11).

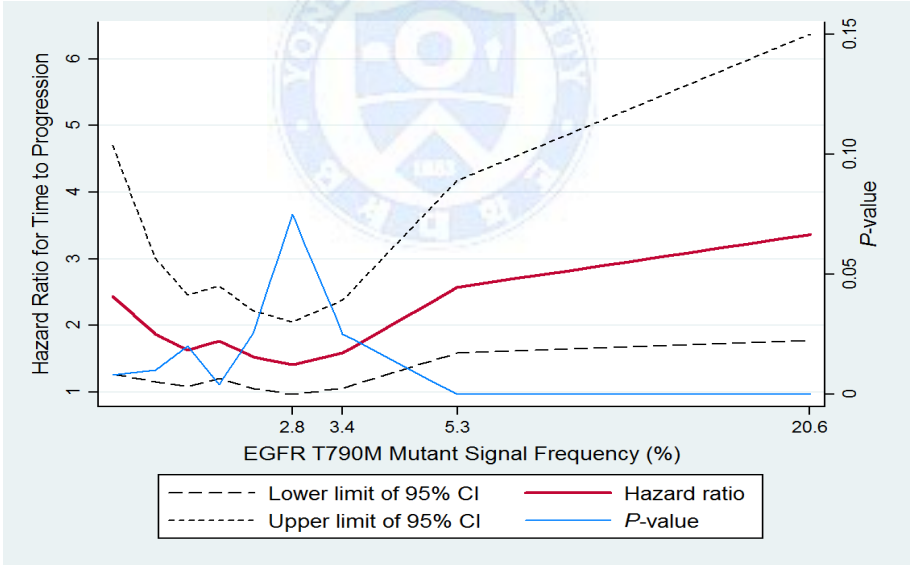

Figure 11. Hazard ratios for TTP following EGFR-TKI therapy according to the cutoff levels of the T790M mutant signal frequency in 124 EGFR-mutant patients. Hazard ratios were calculated using the Cox proportional hazards regression model.

In vitro sensitivity to gefitinib was significantly lower in the cell mixtures containing ≥10% of H1975 cells (TKI-resistant cells) compared with that of pure H827 cells (TKI-sensitive cells) (Figure 12A,B). Additionally, in mixtures of PC9GR-T790M cells and PC9 cells, the minimum percentage of PC9GR-T790M cells that started to show a reduction in gefitinib sensitivity relative to pure PC9 cells was 5% (Figure 12C,D).

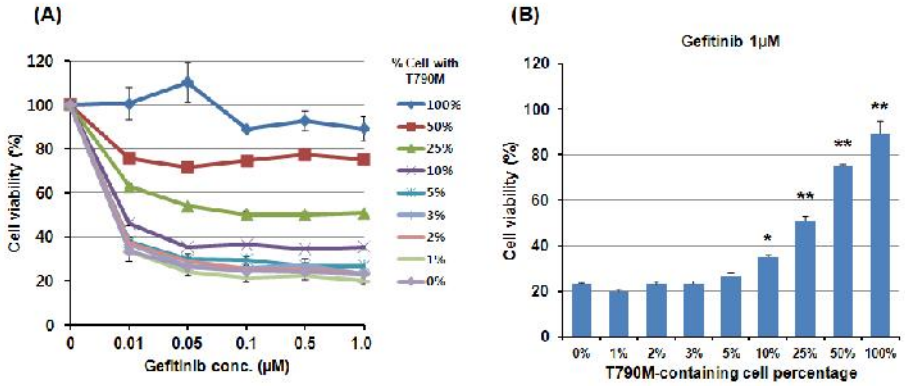

Figure 12. *In vitro* experiments for estimating the percentage of T790M cells with resistance to gefitinib. TKI-sensitive cells (HCC827 and PC9) and TKI-resistant cells with the T790M mutation (H1975 and PC9GR-T790M) were mixed at the indicated proportions and were treated with gefitinib. (A, B) Mixtures of HCC827 and H1975 cells. (C, D) Mixtures of PC9 and PC9GR-T790M cells. *$P < 0.01$ and **$P < 0.001$ (Mann–Whitney U test).

IV. DISCUSSION

The current study semi-quantitatively measured preexisting *EGFR* T790M mutation using the MS-based genotyping assay and ddPCR and evaluated the clinical significance of its level in advanced NSCLC patients with sensitive *EGFR* mutations. These results suggest that the preexistence of resistant subclones before TKI exposure is not rare in tumors harboring TKI-sensitive mutations, but the latent resistant subclones are not always detected by direct sequencing methods because of their low frequency. Similar findings have been reported for the other tumor types, including *KRAS* wild-type colorectal cancer.[21, 22] The latest studies that used deep sequencing methods, either 454 pyrosequencing or BEAMing, revealed that *KRAS* mutations mediate acquired resistance to anti-EGFR treatment in *KRAS* wild-type colorectal cancer and that these alterations exist as latent subclones within the tumor before starting therapy.[21, 22]

As described above, advances in sequencing technology have provided direct evidence for intratumor genetic heterogeneity, suggesting the preexistence of infrequent resistant subclones. However, the impact of these resistant subclones on clinical outcomes, including response and

resistance to therapy, remains to be elucidated. This study revealed that The T790M clones may expand during EGFR-TKI treatment by dying sensitive cell and finally be responsible for the patients' resistance and progression. Thus, the presence of latent resistant clones was associated with reduced survival benefit from molecular targeted therapy. Although patients with preexisting T790M mutations responded to EGFR-TKI therapy, the duration of response was much shorter than that of patients without the T790M mutation. In particular, the clinical outcome of EGFR-TKI therapy was worse in high-leveled T790M patients than in low-leveled T790M patients. Thus, the clinical outcome of patients with *EGFR*-mutant tumors harboring a high proportion of the T790M mutation was as poor as that of patients with wild-type *EGFR*. These results suggest that the negative impact of the T790M mutation on EGFR-TKI efficacy may be dependent on the intratumoral proportion of the T790M mutation.

In this study, we used two different approaches to determine the lowest frequency of the T790M mutations in *EGFR*-mutant tumor that reduces the efficacy of EGFR-TKI therapy. In both sets of cell-line mixture experiments, the mixtures containing 5% or 10% of T790M cells showed significantly decreased sensitivity to gefitinib compared with the

mixture without T790M cells, whereas the mixtures containing less than 5% or 10% of T790M cells showed no significant differences in sensitivity to gefitinib relative to the mixture without T790M cells. In clinical settings, the lowest frequency of T790M cells to affect sensitivity to EGFR-TKI therapy was estimated to be 3.2%. This discrepancy between the clinical and cell-line results may be explained by the difference in the composition of tumor DNA content between the tissue samples and the cell lines. Although we selected samples in which the tumor comprised ≥70% of the sample, the inclusion of non-malignant normal cells, such as stromal and inflammatory cells, possibly resulted in underestimation of the lowest frequency of the T790M mutation in tissue samples unlike in tumor cell lines. It is also possible that different numbers of the T790M allele within each tumor cell could have contributed to the discrepancy.

V. CONCLUSION

Several treatments have been developed to overcome the limitations of the first-generation EGFR-TKIs in *EGFR*-mutant lung cancer and several T790M-specific TKIs are currently being evaluated in clinical trials. Thus, it is important that we understand the clinical significance of the T790M mutation as the major cause of resistance to EGFR-TKI. This study revealed that the presence of preexisting T790M mutation is not rare event and the frequency of the T790M mutation within a tumor has a critical impact on the tumor's biological characteristics and the clinical outcomes of EGFR-TKI therapy. The extent of preexisting *EGFR* T790M mutations is a challenging target for future diagnostic and therapeutic studies of *EGFR*-mutant lung cancer.

REFERENCES

1. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; 350(21): 2129-39.

2. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352(8):786-92.

3. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Medicine* 2005;2(3):e73-e73.

4. Yun C, Mengwasser KE, Toms AV, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci U S A.* 2008;105(6):2070-75.

5. Yu HA, Arcila ME, Rekhtman N, et al. 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* 2013;19(8):2240-47.

6. Mitsudomi T, Morita S, Yatabe Y, 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 oncology* 2010; 11(2):121-28.

7. Zhou C, Wu Y, Chen G, 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 oncology* 2011; 12(8):735-42.

8. Rosell R, Carcereny E, Gervais R, 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 oncology* 2012; 13(3):239-46.

9. Shih J, Gow C, Yang P. EGFR mutation conferring primary resistance to gefitinib in non-small-cell lung cancer. *N Engl J Med* 2005; 353(2):207-08.

10. Inukai M, Toyooka S, Ito S, et al. Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. *Cancer Res* 2006;66(16):7854-58.

11. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med* 2008;

359(4):366-77.

12. Tanaka T, Matsuoka M, Sutani A, et al. Frequency of and variables associated with the EGFR mutation and its subtypes. *Int J Cancer.* 2010;126(3):651-55.

13. Su K, Chen H, Li K, et al. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. *J Clin Oncol.* 2012;30(4):433-40.

14. Fujita Y, Suda K, Kimura H, et al. Highly sensitive detection of EGFR T790M mutation using colony hybridization predicts favorable prognosis of patients with lung cancer harboring activating EGFR mutation. *J Thorac Oncol.* 2012;7(11):1640-44.

15. Fukuoka M, Wu Y, Thongprasert S, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). *J Clin Oncol.* 2011;29(21):2866-74.

16. Costa C, Molina MA, Drozdowskyj A, et al. The impact of EGFR T790M mutations and BIM mRNA expression on outcome in patients with EGFR-mutant NSCLC treated with erlotinib or chemotherapy in the

randomized phase III EURTAC trial. *Clin Cancer Res* 2014; 20(7):2001-10.

17. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92(3):205-16.

18. Cardarella S, Ortiz TM, Joshi VA, et al. The introduction of systematic genomic testing for patients with non-small-cell lung cancer. *J Thorac Oncol* 2012;7(12):1767-74.

19. Johnson ML, Sima CS, Chaft J, et al. Association of KRAS and EGFR mutations with survival in patients with advanced lung adenocarcinomas. *Cancer* 2013;119(2):356-62.

20. Chmielecki J, Foo J, Oxnard GR, Hutchinson K, Ohashi K, Somwar R, et al. Optimization of dosing for EGFR-mutant non-small cell lung cancer with evolutionary cancer modeling. *Sci Transl Med.* 2011;3(90):90ra59-90ra59.

21. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012;486(7404):532-36.

22. Diaz LA, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 2012;486(7404):537-40.

23. Oxnard GR, Arcila ME, Sima CS, Riely GJ, Chmielecki J, Kris MG, et al. Acquired resistance to EGFR tyrosine kinase inhibitors in EGFR-mutant lung cancer: distinct natural history of patients with tumors harboring the T790M mutation. *Clin Cancer Res.* 2011; 17(6):1616-22.

24. Rosell R, Molina-Vila MA, Taron M, Bertran-Alamillo J, Mayo C, Vergnenegre A, et al.: EGFR compound mutants and survival on erlotinib in non-small cell lung cancer (NSCLC) patients (p) in the EURTAC study. *2013 ASCO meeting* 2012:Abstract 7522.

25. Prudkin L, Tang X, Wistuba I. Germ-line and somatic presentations of the EGFR T790M mutation in lung cancer. *J Thorac Oncol.* 2009;4(1):139-41.

26. Tibaldi C, Giovannetti E, Vasile E, Boldrini L, Gallegos Ruiz MI, Bernardini I, et al. Inherited germline T790M mutation and somatic epidermal growth factor receptor mutations in non-small cell lung cancer patients. *J Thorac Oncol.* 2011;6(2):395-96.

27. Girard N, Lou E, Azzoli CG, Reddy R, Robson M, Harlan M, et al.

Analysis of genetic variants in never-smokers with lung cancer facilitated by an Internet-based blood collection protocol: a preliminary report. *Clin Cancer Res* 2010;16(2):755-63.

ABSTRACT(IN KOREAN)

EGFR 타이로신 키나아제 저해제에 민감한 *EGFR* 유전자 돌연변이를 가진 폐암에서 잠복 내성 클론의 존재 확인 및 특성 규명

<지도교수 장 준>

연세대학교 대학원 의학과

이영주

EGFR 타이로신 키나아제 저해제는 폐암 치료의 패러다임을 바꾼 최초의 분자 표적 항암 치료제이다. 이 약물은 민감성 *EGFR* 유전자 돌연변이를 가진 폐암 환자들에서만 특징적으로 효과가 높다. 그러나 초기에 이 약물에 대한 반응이 좋았던 환자들도 결국에는 약물 내성을 보이고 마는데 이 약물 내성의 원인 중에 가장 많은 부분을 차지하는 것이 EGFR 유전자의 T790M 돌연변이이다. 본 연구는 이러한 T790M 돌연변이를 가진 암세포가 약물에 노출되기 이전부터 존재하면서 약물에 노출된 후 그 수가 증가하는 지를 알아보았다. Sanger 시퀀싱 방법으로 민감성 EGFR 돌연변이의 존재가 확인된 진행성 비소세포폐암 환자 124명에서 치료 전 종양 샘플을 모아서 matrix-assisted laser desorption/ ionization-time of flight/ mass spectrometry를 통하여 T790M의 유전형을 확인했을 때 31명 (25%)에서 T790M 돌연변이를 확인할 수 있었고 이차 분석을 위하여 종양 샘플이 남아 있는 68명에서 droplet digital PCR 통하여 다시 확인했을 때 27명 (39.7%) 에서 T790M 돌연변이를 확인할 수 있었다. 본 연구에서는 또한 약물 치료 동안 T790M 클론의 확장을 In vivo 모델과 치료 전후 환자 샘플을 이용하여 확인할 수 있었다. 약물 민감성 세포와 luciferase가 표지 된

약물 내성 세포를 함께 배양했을 때 약물 내성 세포는 약물 민감성 세포가 약물에 의하여 죽어갈 때 특히 잘 성장함을 발견하였다. T790M 돌연변이의 종양 내 수가 3.2%를 넘어설 때 이 약물에 대한 효과가 떨어질 위험도가 높아지기 시작하였다. T790M 양성 종양을 가진 환자는 T790M 음성 환자보다 짧은 무진행 생존 기간 및 총 생존 기간을 가졌다. 게다가, T790M 양성 환자 중에 T790M 돌연변이 분포가 높은 환자는 낮은 환자와 비교할 때 짧은 무진행 생존 기간 및 총 생존 기간을 가졌다. 결론적으로, EGFR 양성 폐암 환자에서 T790M 돌연변이는 상당량 존재하고 이 돌연변이를 가진 암세포는 주위의 T790M 돌연변이가 없는 약물 민감성 세포가 약물로 인해 죽어감에 영향을 받아서 성장이 촉진됨을 확인할 수 있었다. 이로 인해 T790M 돌연변이 분포가 높은 환자는 이 표적 치료제를 사용함에도 불구하고 불량한 예후를 보였다.

핵심되는 말: 폐암, 표적 치료, EGFR, 타이로신 키나아제 저해제, 후천성 내성, EGFR T790M

PUBLICATION LIST

1. Lee Y, Lee GK, Zhang W, et al. Clinical outcome according to the level of preexisting epidermal growth factor receptor T790M mutation in patients with lung cancer harboring sensitive epidermal growth factor receptor mutations. *Cancer* 2014;120:2090-2098.
2. Lee Y, Lee GK, Hwang J, et al. Clinical likelihood of sporadic primary EGFR T790M mutation in EGFR-mutant lung cancer. *Clinical lung cancer* 2015;16:46-50.