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**Title:** The *EGFR* T790M mutation in acquired resistance to an irreversible second-generation EGFR inhibitor

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## Abstract

Molecular target therapies using the first-generation reversible epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib or erlotinib, have been shown to be effective for non-small cell lung cancer (NSCLC) patients who harbor activating mutations in EGFR. However, these patients eventually develop resistance to the reversible TKIs, and this has led to the development of second-generation irreversible EGFR inhibitors. Currently, the mechanism of acquired resistance to irreversible EGFR inhibitors is not clear. Using an *in vitro* cell culture system, we modeled the acquired resistance to first-line treatment with second-generation EGFR-TKIs, using an *EGFR* mutant NSCLC cell line. Here we report a mechanism of resistance involving T790M secondary mutation as well as a corresponding clinical case. The results of these findings suggest that inhibition of EGFR by currently available second-generation EGFR-TKIs may not be sufficient to physiologically prevent the emergence of cells that are still dependent on EGFR signaling. This finding bears important implications on the limitations of currently available second-generation EGFR TKIs.

## Introduction

The successful clinical usage of imatinib seen in the case of chronic myeloid leukemia patients has led to the notion that some cancer cells are dependent on a limited number of oncogenic pathways, and that tumors can be controlled by specific inhibition of these critical pathways (1-3). This notion has been further expanded to the case of solid tumors, where administration of EGFR tyrosine kinase inhibitor (TKI)s has been shown to produce a dramatic clinical response in a subset of lung cancer patients (4-6).

It was subsequently observed that the majority of lung cancer patients responsive to EGFR-TKIs also harbor activating mutations in the EGFR tyrosine kinase domain (4-6). This further bolsters the notion that searching for genetic signatures related to oncogenic alterations could serve as predictive biomarkers for the corresponding molecular target inhibitors. In contrast to the case of CML, however, the efficacy of EGFR-TKI in lung cancer was found to be severely compromised by the rapid emergence of targeted therapy-resistant clones; most lung cancer patients receiving EGFR-TKI therapy acquire resistance within one year. Detailed molecular analyses have shown that about half of these patients develop resistance through a compound T790M *EGFR* substitution mutation, placed in *cis* with a primary activating mutant *EGFR* allele (7-10).

Efforts to block signaling from the compound mutant *EGFR* with small molecule inhibitors has led to the conceptual introduction of experimental irreversible EGFR-TKIs that function primarily by covalently attaching to the cysteinyl-797 residue in the pocket of the EGFR kinase domain (11). These compounds have been shown to be modestly efficacious in coping with the acquired resistance due to the T790M mutation in pre-clinical *in vitro* model studies (12). Further pharmacological efforts led to the development of second generation EGFR-TKIs, such as HKI-272, BIBW2992 (afatinib) and PF00299804. Various *in vitro* and *in vivo* pre-clinical animal model studies supported the experimental observation that irreversible EGFR-TKIs could be potentially effective in blocking T790M EGFR-derived signaling pathways (13-17).

Second generation irreversible EGFR-TKIs are currently being tested in clinical settings as alternative therapeutic options for patients who acquired resistance to gefitinib or erlotinib via the T790M mutation (18). It has been shown that the covalent attachment of certain irreversible EGFR-TKIs to the kinase pocket is largely non-discriminatory between the active

conformation of wild-type EGFR and that of the mutant, providing rationale for the use of these molecules in overcoming the enzymatic kinetics problem associated with the restored ATP-affinity of T790M EGFR (19, 20). This non-discrimination, though effective in overcoming kinetics problem of T790M, raises concern in terms of applicability in clinical settings, given that non-discriminatory blockade of wild-type and mutant *EGFR* could eventually limit the effectiveness due to issues related to side-effects. Thus, applicability of this compound in clinics will be largely dictated by the effective serum concentration of this class of drugs, which must meet the dual requirement of tolerability for the patients and functionality against tumors.

In this study, we modeled the treatment with the irreversible EGFR-TKI, BIBW2992 (afatinib), to gefitinib- or erlotinib-naïve *EGFR* mutant lung cancer and generated the cells that acquired the trait of resistance to irreversible EGFR-TKIs. The analysis of the resulting clones provides insights into the pharmacological mechanistic basis underlying the requirement for alternative treatment schemes.

## Materials and Methods

### Cell culture and drug treatments

NSCLC PC9 cells (exon 19 del E746-A750) were kindly provided by Dr. K. Nishio (National Cancer Center Hospital, Tokyo, Japan). This cell line was extensively characterized previously (21-24) and it was repeatedly tested in the laboratory for its authentication by genotyping and morphological observation. PC9 cells or their derivatives were verified by morphology and growth curve analysis, and were tested for *Mycoplasma*. PC9 NSCLC cells were maintained in RPMI 1640 (GIBCO) with 10% fetal bovine serum (GIBCO) and 1% antibiotic-antimycotic (GIBCO) in a 37°C incubator. BIBW2992 was kindly provided from Boehringer Ingelheim. PC9TR3 cells were generated under the continuous stress of erlotinib treatment. The Sanger sequencing of the PC9TR3 EGFR TK domain revealed the presence of the T790M mutation (data not shown).

### Western blotting and antibodies

Cells were harvested, washed with PBS, and lysed in lysis buffer [50 mmol/L Tris (pH 7.4), 1% Triton-X, 150 mmol/L NaCl, 40 mmol/L NaF, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 10% glycerol, 1 mmol/L phenylmethylsulfonylfluoride, and 10 µg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor] for 30 min on ice. Cell lysates were centrifuged at 14,000 rpm for 15 min, and the protein concentrations were determined via bicinchoninic acid assay (Pierce Biotechnology). Total protein (60 µg) was resolved by SDS-PAGE and transferred onto PVDF. Blots (with 5% BSA) were probed with antibodies overnight at 4°C to detect the protein of interest. The antibodies used for Western blotting were EGFR antibody (Cell Signaling Technology), phosphor-EGFR (Cell Signaling Technology, Tyr1068), p-ErbB3 (Cell Signaling Technology), p-STAT3 (Cell Signaling Technology), STAT3 (Cell Signaling Technology), AKT (Cell Signaling Technology), phosphor-AKT (Cell Signaling Technology, Ser473), ERK (p44/42 MAP kinase, Cell Signaling Technology), phosphor-ERK (p44/42 MAP kinase, Tyr202/Tyr204, Cell Signaling Technology), actin and actinin (Santa Cruz), phosphor-p70S6kinase (Cell Signaling Technology), PTEN (Cell Signaling Technology), BIM (Cell Signaling Technology), phosphor-cMET (Invitrogen Biosource, 44887G), cMET (Santa Cruz), phospho-IGF-1R (Cell Signaling Technology), and IGF-1R (Cell Signaling Technology).

### **MTT cell viability**

To measure the sensitivities to anti-cancer drugs, a methylthiotetrazol (MTT) assay was performed. In brief, PC9 cells ( $5 \times 10^3$ /well) were seeded onto 96-well plates and were pre-incubated overnight. The cells were continuously exposed to the indicated concentrations of drugs with 1% FBS for three days. Absorbance was measured at 540 nm with a microplate reader (Molecular Device, 384 plus).

### ***In vivo* study**

All animals were maintained in a facility at Samsung Biomedical Research Institute (SPF, specific pathogen free) in accordance with institutional guidelines.  $1 \times 10^6$  PC9 or PC9 BIBW2992-resistant cells were inoculated onto balb/c nude mice (n=4). Daily oral doses of BIBW2992 (35 mg/kg) in captisol solution were administered to tumor-bearing mice. Tumor size was measured twice a week to follow the drug response in animal model studies.

### **Reagents and constructs**

EGFR siRNA (sense 5'cagaggauguucauaacu3' antisense 5'aguauugaacauccucu3' UU overhang) and control siRNA (sense 5'gagcgaucauguauaauc3' antisense 5'ugauuauacuagaucgcuc3' UU overhang) were ordered from Bioneer (Daejeon, Korea). *c-met* copy number was measured by SYBR Green-based real-time PCR assay with *GAPDH* as the normalization control. Gene dosage of the T790M allele was determined using molecular beacon-based fluorescence detection. The detailed protocol is described in Oh *et al.* (25). The EGFR T790M compound mutant constructs were generated by point mutagenesis of EGFR activating mutant constructs. They were expressed in PC9 cells, using retroviral transduction with pBabe vectors and drug-selected with puromycin.

### **EGFR sequencing**

EGFR exons from PC9BR clones were sequenced from genomic DNA by conventional Sanger sequencing. In the case of a patient sample, tumor biopsy specimens from a patient with an activating *EGFR* mutation, who had been treated with BIBW2992, were obtained from the U.C. Irvine Medical Center (Orange, CA, USA) as part of institutional review board-approved studies. All patients provided written informed consent. The *EGFR* exons

were amplified and sequenced or independently assayed by Genzyme Corporation (Cambridge, MA, USA).

## Results

### Establishment of BIBW2992-resistant PC9 lung cancer cells

In order to establish an *in vitro* cell line model system for acquired resistance to the first-line molecular target therapy involving afatinib treatment, we chose to use NSCLC PC9 cells that contained an *EGFR* exon 19 in-frame deletion (delE746-A750). These cells demonstrate exquisite sensitivity to EGFR-TKIs, including gefitinib and erlotinib, within nanomolar drug concentration range (Fig. 1A). A previous study has shown that prolonged treatment of these cells with gefitinib or erlotinib eventually led to the emergence of clones harboring the T790M secondary mutation (unpublished data), which confers acquired resistance to reversible EGFR-TKIs (Fig. 1A). We hypothesized that next-generation irreversible EGFR-TKIs might possess the capacity to effectively block the emergence of T790M secondary mutant clones, thereby completely blocking or significantly delaying the appearance of resistant mutants in model system studies.

A prototypical *in vitro* dose-escalation study employing a molecular target inhibitor was adopted (26, 27) to establish BIBW2992-resistant (BR) PC9 cells. PC9 cells were initially cultured in the presence of a low concentration of afatinib (~10nM) in the beginning, and then were dose-escalated for four months. After prolonged treatment with afatinib, several independent afatinib-resistant clones emerged that persisted and proliferated even in the presence of 2  $\mu$ M afatinib. From these, we established 14 different independent clones that acquired resistance to afatinib. Some of the drug responses of the representative resistant clones are shown in Fig. 1B. To monitor whether the acquisition of resistance was irreversible, we cultured all of these clones in normal growth medium without the RTK inhibitor. These clones remained resistant to afatinib after incubation in normal growth medium for several months (data not shown), indicating that these cells underwent an irreversible change, possibly involving chemically stable physical alterations in their genetic space. The signature exon 19 deletion (del E746-A750) of parental PC9 cells remained unaltered in all of the resistant clones.



## Classification of EGFR-TKI-resistant cells

To test whether these TKI-resistant clones were still dependent on EGFR, we monitored the effect of EGFR depletion via siRNA-mediated EGFR knock-down. Two different types of EGFR dependence were noted among the BIBW2992-resistant clones. One subgroup of resistant clones displayed continued dependence on EGFR expression, as evidenced by their markedly decreased cell proliferation and/or cell survival upon depletion of the *EGFR* transcript (Fig. 2A; clones #1, 2, 3, 6, 8, 11, 12, 13, 14, 15, 16). The other subgroup of resistant cells, however, demonstrated a substantially decreased requirement of EGFR signaling (Fig. 2A; clones #4, 5, 10). The decrease in EGFR expression in these clones did not significantly affect the cell proliferation rate of the clones, and the cells grew at a rate almost comparably to that of the control siRNA-treated samples, suggesting that these cells acquired TKI-resistance by escaping EGFR dependence.

Given the dichotomy of response profiles related to EGFR dependence, we next set out to examine the presence of a possible correlation between the EGFR dependence of clones and their *EGFR* kinase domain sequence alterations. We sequenced *EGFR* kinase domain exons 18-21 to examine the primary sequences of the *EGFR* tyrosine kinase domain in the resistant clones. The EGFR-independent clones (Fig. 2B, e.g. #10) showed no genetic alterations in their *EGFR* kinase domain. In stark contrast, we found that all of the EGFR-dependent resistant clones invariably shared a common *EGFR* exon 20 T790M mutation, with the signature chromatographic peak robustly detectable through classical Sanger sequencing (Fig. 2B, e.g. #6; Supplementary Info. 1A-B). To check whether a subset of these resistant clones developed resistance by *c-met* gene amplification, we performed real-time quantification of *c-met* gene copy number and found no statistically significant evidence of such a genetic change (Supplementary Info. 2A). In addition to *c-met* amplification, *PTEN* deletion was reported to be involved in resistance to erlotinib (28). PTEN protein expression of afatinib-resistant clones, however, was found to be comparable to that of parental PC9 cells (Supplementary Info. 2B). No morphological alteration, indicative of epithelial-mesenchymal transistion, was notable among resistant cells.

To see how the downstream EGFR signaling networks of BIBW2992-resistant cells respond to the challenge of the irreversible EGFR-TKI, we examined protein expression and

modification profiles over a range of afatinib concentrations. Treatment of the resistant clones with afatinib affected RTK activation and its downstream signaling networks in a markedly different way, according to EGFR dependence. In the EGFR-independent cells, EGFR C-terminal phosphorylation was effectively shut down by afatinib, comparable to the effect seen in parental PC9 cells. On the other hand, resistant cells containing T790M demonstrated a clearly reduced sensitivity to afatinib, based on assessment of their EGFR C-terminal phosphorylation (Fig. 2C).

As expected, due to the escape from EGFR dependence, the activation of downstream signaling networks in EGFR-independent clones was uncoupled from upstream EGFR activation status, which manifested as high-level AKT and ERK signaling pathway activation regardless of EGFR phosphorylation status. EGFR-dependent clones demonstrated a strictly coordinated EGFR activation profile between the receptor and downstream signaling networks. Thus, when challenged with the highest dose of afatinib, they demonstrated a complete shutdown of EGFR activation, followed by concurrent blockade of downstream signaling networks (Fig. 2C).

To test the effect of the T790M mutation on the sensitivity of afatinib, a retroviral mutant *EGFR* construct containing T790M compound mutation was transduced into PC9 cells. Compared to control vector-transduced cells, the ectopic expression of the T790M mutant markedly altered the sensitivity to afatinib (Fig. 2D). This confirms the previous conclusion, obtained from the loss-of-function experiments via siRNA, that the T790M allele is the main factor that significantly abrogates the efficacy of afatinib.

#### Gene dosage of T790M-containing allele correlated with the degree of resistance

More careful examination of the afatinib-resistant clones indicated that there were two classes of resistant clones within the EGFR-dependent subset of T790M-containing cells. One group displayed stronger afatinib *in vitro* resistance than the other (e.g. Fig. 3A). To assess whether the gene dosage of the T790M-containing allele might be a factor in determining the degree of resistance, we determined the level of the T790M allele, using genetic variation specific molecular beacon-based real-time quantification (25). Some T790M-containing clones had more copies of the T790M allele and demonstrated resistance to afatinib more robustly *in vitro*, suggesting that the gene dosage of the T790M allele is

another factor in determining the degree of drug resistance (Fig 3B, Supplementary Info. 1A-B). Different gene dosage of the T790M allele also affects the drug response and protein modification profile accordingly (Supplementary Info. 3).

#### Recapitulation of a resistance phenotype seen in a xenograft animal model

To conclusively determine if cells obtained from the *in vitro* selection process also phenocopy the resistance traits in animal model studies, we transplanted representative BIBW2992-resistant clones into nude mice. These BIBW2992-resistant PC9 tumor-bearing mice received an oral maximum tolerance dose (MTD) of afatinib. Consistent with *in vitro* and biochemical profiles, BIBW2992-resistant clones demonstrated a robust resistance phenotype *in vivo* compared to parental PC9 cells (Fig. 3C). It is of note that, in contrast to the results from the *in vitro* cell culture data, the low T790M-containing clone #1 also displayed a similar degree of *in vivo* resistance to afatinib administration at its maximum tolerated dose.

#### Case report of a lung cancer patient treated with afatinib

To assess the clinical impacts of these *in vitro* and animal model studies, we analyzed the lung cancer samples of a patient, who had been treated with afatinib. The patient was a 44-year-old female who had never smoked and who had received a diagnosis of stage IIIA lung adenocarcinoma (Fig. 4A, clinical information available in Supplementary Info. 4). EGFR sequencing of the resected tumor sample revealed the L747\_P753 *del.* 17 mutation in exon 19. This patient, not previously treated with gefitinib or erlotinib, was enrolled in a Phase II study with afatinib at a 40mg dose. Upon start of drug administration, cancer nodules of the patient responded well to the treatment (Fig. 4B). Eventually, however, she developed a recurrent metastatic nodule that initially responded to afatinib. Wedge resection of the resistant pulmonary nodule revealed the presence of the T790M mutation in the EGFR TK domain, which was not present in the pre-treatment tumor tissue (Fig. 4C). The presented clinical case suggests that prolonged afatinib treatment eventually can lead to the emergence of acquired resistance and the mechanism of resistance observed in clinics also involves the emergence of the EGFR T790M mutation, as predicted from the model system studies.

## Discussion

The lack of a reliable model system to predict clinical response of gefitinib- or erlotinib-naïve *EGFR* mutant lung cancers under continuous challenges with irreversible EGFR-TKIs, including afatinib, prompted the current study with the goal of making relevant predictions in ongoing clinical trials. To establish the *in vitro* model of acquired resistance to first line usage of afatinib, we utilized NSCLC cells possessing an EGFR tyrosine kinase domain activating mutation. EGFR activating mutant NSCLC cells cultured under the stress of continuous presence of afatinib in culture medium eventually produced several clones that were resistant to irreversible EGFR-TKIs. Unexpectedly, analysis of clones dependent on EGFR signaling contained the same genetic signature (T790M), which was shared by clones resistant to gefitinib or erlotinib. siRNA-mediated EGFR depletion culminated in rapid cell death of these clones, strongly suggesting that this altered sequence signature in the EGFR TK domain is likely responsible for mediating resistance to the irreversible EGFR-TKI. This finding raises the concern that the first line usage of currently available irreversible EGFR-TKIs may not be mechanistically distinct or qualitatively superior in preventing cancer cells from acquiring a resistance phenotype.

Thus, in contrast to transgenesis-based animal model studies (16), where animals might be able to tolerate higher doses, clinical usage of this drug might not be as effective as anticipated. On the other hand, it should also be noted that a similar transgenic animal model failed to validate the monotherapeutic efficacy of afatinib against EGFR L858R/T790M mutation (29). The primary reason why this drug is less effective in controlling T790M mutant *EGFR* in cancer signaling seems to be related to the non-discriminatory action of this compound toward active conformation of wild-type *EGFR* and that of mutation (12). Indeed, the recent screening of T790M mutation-specific small molecule-based inhibitors suggests that oncogenic mutant tailored drug design may overcome part of the problem related to insufficient discriminatory power and consequently less efficient action of molecular target therapies (30) and clinical trials with the mutant-specific inhibitors are under development.

Previous studies employing HKI-272, another irreversible EGFR-TKI, have shown that EGFR-dependent lung cancers become resistant to HKI-272 in similar manners (31). Though this study is one of the first studies providing insightful evidence to support the potential

limitations of currently available irreversible EGFR-TKIs, the context of the study was highly artificial in that they used a mutagen, ethyl methane sulfonate, to induce resistance to EGFR-TKIs (31). In contrast, the BIBW2992-resistant clones obtained in the current study arose under the natural stress of afatinib, thus mimicking a more physiologically relevant context. Additionally, HKI-272 was tailored to fit into the inactive conformation of EGFR TK (19). Since activating EGFR mutant oncogenic variants, including L858R and exon 19 small in-frame deletions, assume constitutively active kinase conformations (19, 20), it is anticipated that HKI-272 will not function as an effective inhibitor against primary activating mutant EGFRs. Consistent with the results obtained from structural studies (19, 20), the  $IC_{50}$  values of inactive conformation fitting drugs (e.g. HKI-272) and active conformation fitting drugs (e.g. BIBW-2992) vary widely between the two drugs in EGFR-dependent PC9 cells (HKI-272  $IC_{50}$ =96nM; afatinib  $IC_{50}$ =0.7nM) (e.g., (30, 32)). Thus, the rationale for utilizing the inactive conformation-fitting EGFR-TKIs to EGFR mutant lung cancer patients is subject to scientific controversy, and this is in line with the decrease in activity seen in clinical trials with HKI-272 (personal communications, (18), (33)).

Although the current set of experimental data suggests that second generation irreversible EGFR-TKIs may be less effective than previously thought at blocking the emergence of T790M mutant-based resistant clones (11), it has not quantifiably addressed the issue of progression-free duration until drug resistance. Ercan *et al.* (17) demonstrated that amplification of the pre-existing T790M locus confers resistance against PF00299804, another irreversible EGFR-TKI, to previously gefitinib-treated and gefitinib-resistant PC9 GR cells. The current study shows that first-line usage of afatinib to gefitinib or erlotinib-naïve EGFR mutant cells also leads to resistance to irreversible EGFR-TKIs via the T790M mutation, a rather unexpected consequence that has been supported by a clinical case as well as the cell-based modeling studies. It should be noted, however, that compared to gefitinib or erlotinib, the emergence and amplification of the T790M locus in EGFR may conceivably take longer in the case of second-generation irreversible EGFR-TKIs. Thus, it still remains possible that patients may benefit from the usage of afatinib in the form of prolonged progression-free survival as their first-line treatment option. Recent phase III clinical studies involving afatinib versus placebo also showed that patients who progressed after 12 weeks of first-generation EGFR-TKIs still benefit from using afatinib in the form of increased progression-free survival (median, 3.3 months vs. 1.1 months;  $P<0.001$ ), though their overall

survival did not differ significantly (34). Several clinical trials are ongoing to demonstrate the duration-related clinical benefit from the usage of second generation EGFR-TKIs in the first-line treatment (e.g. NCT01074177, <http://clinicaltrials.gov>).

The existence of EGFR-dependent as well as independent BIBW2992-resistant clones arising from the same *in vitro* environmental stress indicates that the genetic networks of lung cancer cells responding to an external stress may morph differentially into distinctly adapted versions. The adaptation process seems plastic and stochastic, while some pre-deterministic factors may be involved in other cases (35). The molecular mechanism of escaping EGFR dependence under TKI challenge is being studied and preliminary data suggest a couple of clinical applications. Thus, efforts to determine the genetic causes of drug resistance at various points during the clinical course are necessary to better guide optimal treatment schemes in individual patients.

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## Figure Legends

### Figure 1

Drug response of PC9 or derivative cells *in vitro*. (A) PC9 or PC9 erlotinib (tarceva)-resistant (TR) cells were treated with increasing concentrations of erlotinib and viable cell numbers were analyzed with MTT assay. PC9TR3 cells, shown in the graph, harbor an EGFR T790M secondary mutation. (B) PC9 cells or representative PC9 BIBW2992-resistant (BR) clones were treated with BIBW2992 and their drug response was analyzed with MTT assay. The chemical structure of BIBW2992 (afatinib) is shown under the graph.

### Figure 2

Characterization of PC9BR cells (A) PC9BR clones were transfected with EGFR siRNA and the cell numbers were counted 3 days later. The decrease in cell proliferation is seen in parental PC9 and a subset of PC9BR clones, but not in others (#4, 5, 10). Representative siRNA knock-down of EGFR assessed by Western blotting is shown (siScram, scrambled control siRNA; siEGFR, EGFR siRNA). (B) *EGFR* exon 20 was PCR-amplified from genomic DNA of PC9 BR cells and sequenced. Chromatograms encompassing the region containing *EGFR* T790M are shown. Red circles indicate a C to T substitution, corresponding to T790M. BR#6 contained the T790M mutation, whereas BR#10 did not. (C) Protein profiling of PC9-BR clones. Cells were treated with the indicated amount of BIBW2992 for

24 hours and protein lysates were analyzed by Western blotting. Activation of EGFR and downstream signaling pathways was probed with the indicated antibodies. (D) Drug sensitivity of PC9 cells, retrovirally transduced with either control or EGFR exon19del T790M vector. Cell viability was visualized with MTT assay.

### Figure 3

T790M allele dosage and *in vivo* xenograft animal model. (A) The drug response of representative PC9BR clones is shown, as assessed by MTT assay. (B) The T790M level was semi-quantified using a T790M-specific molecular beacon real-time probe. gDNA from PC9 parental cells (negative control), H1975 cells (positive control for the presence of EGFR T790M), and representative PC9BR clones including #1, #6 and #10 was extracted and used as template in the molecular-beacon real-time detection system. (C) PC9 BIBW2992-resistant clone xenograft tumor model *in vivo*.  $1 \times 10^6$  cells were injected s.c. into nude mice with randomization. Treatment with afatinib (35mg/kg p.o. daily for 4 weeks) was initiated once the tumors achieved a volume of 50 mm<sup>3</sup>. Bar, SE.

### Figure 4

Tumor recurrence associated with the emergence of T790M mutation. (A) A lung adenocarcinoma patient who had a EGFR exon 19 deletion mutation was treated with afatinib. (B) Initially, the two nodules responded to the afatinib treatment. (C) A recurrent metastatic nodule was detected after prolonged afatinib treatment. Analysis of the recurrent nodule showed the presence of the T790M mutation, which was not present in the original lung cancer specimen.

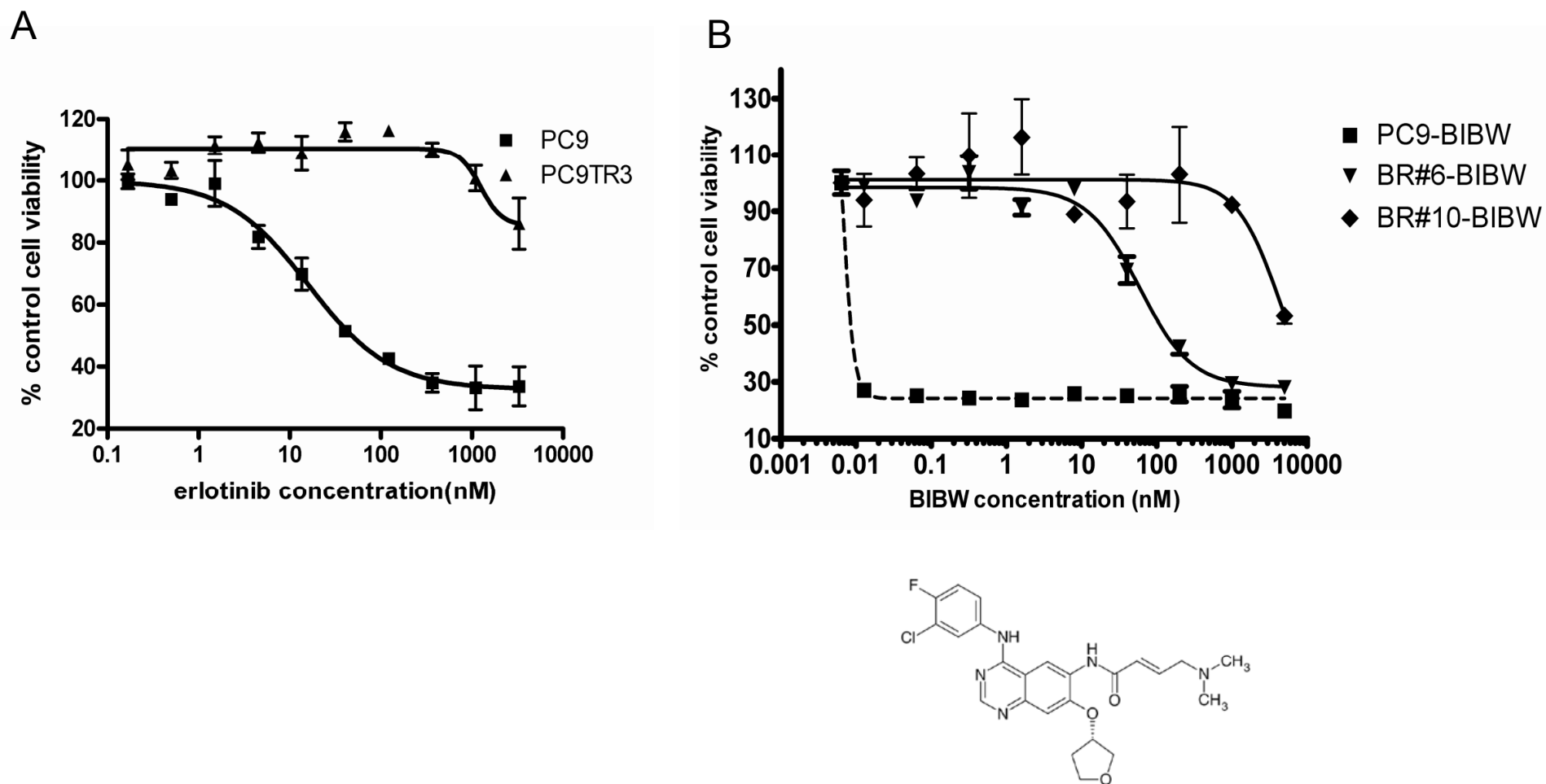
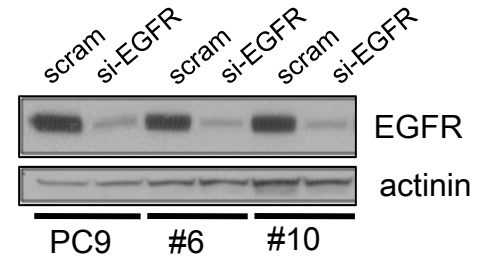
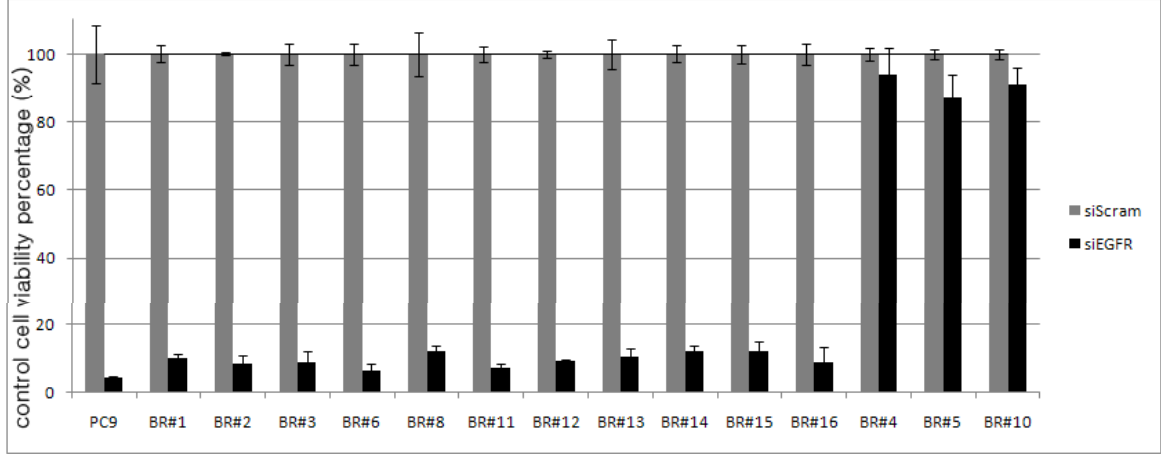
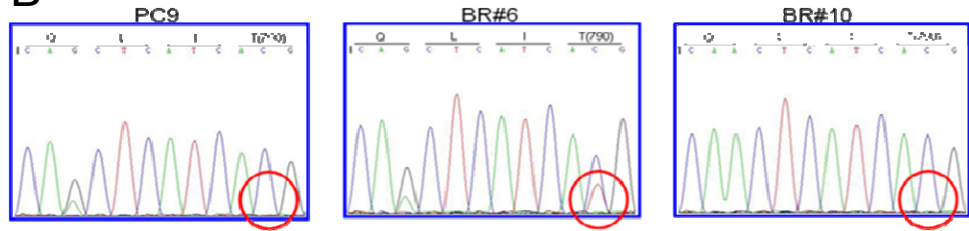


Fig.1

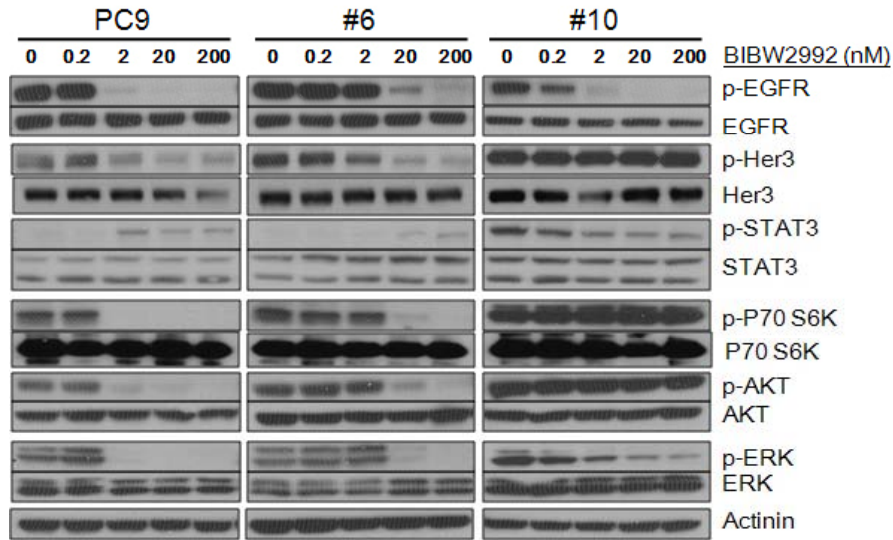
A



B



C



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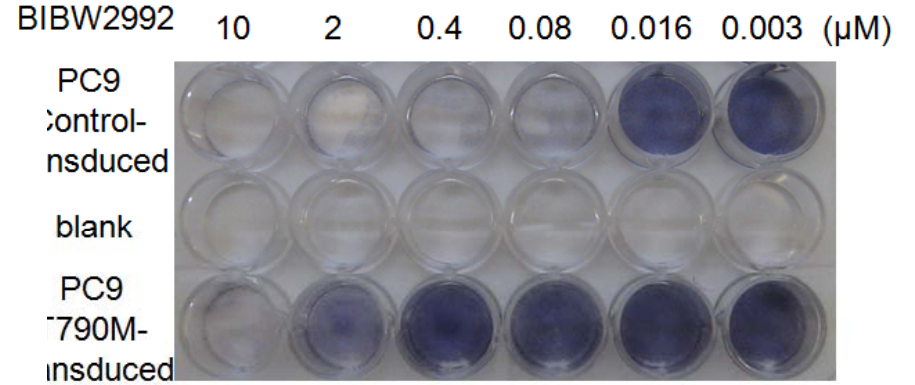
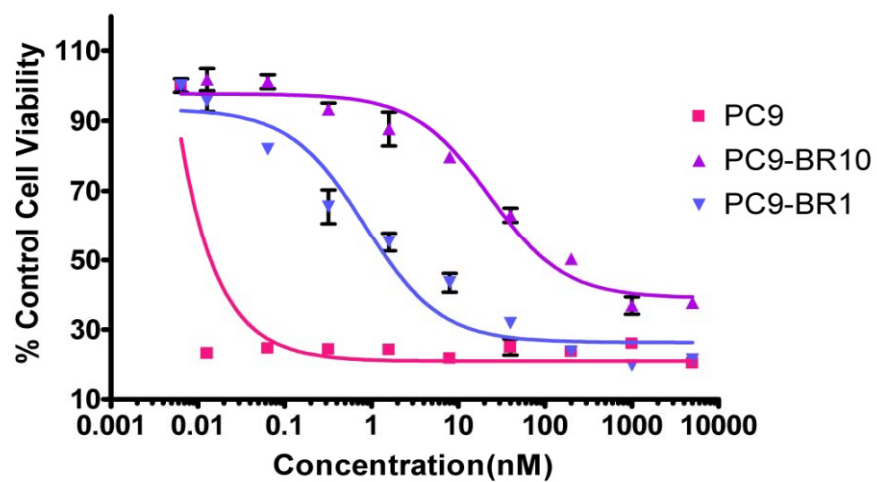
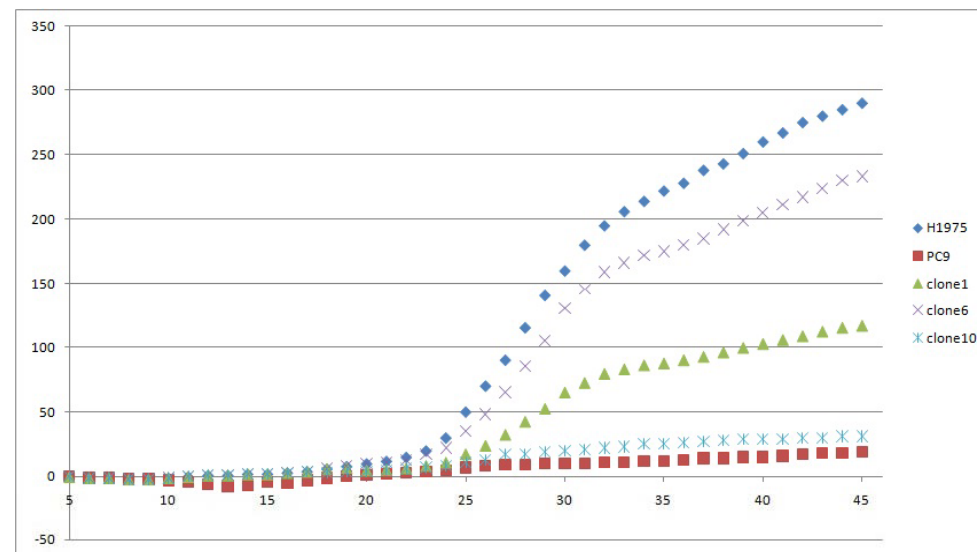


Fig.2

A



B



C

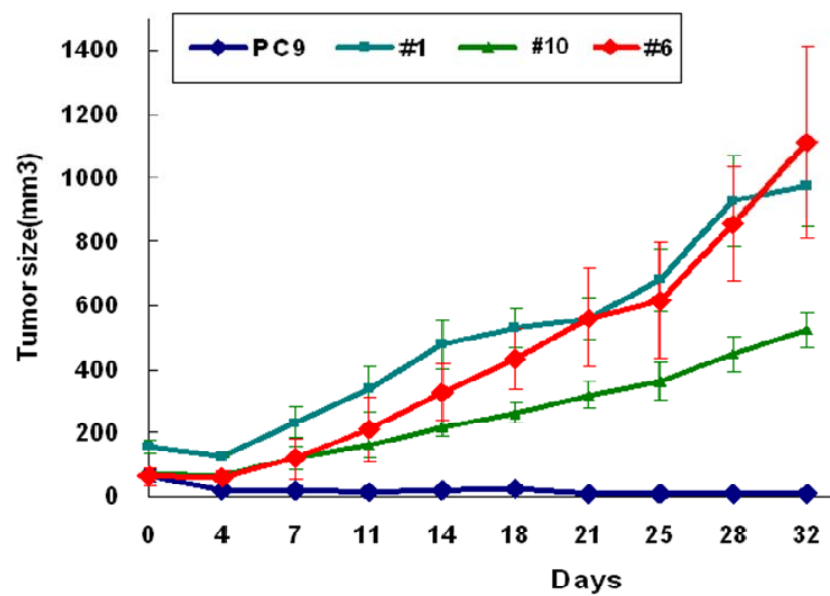


Fig.3

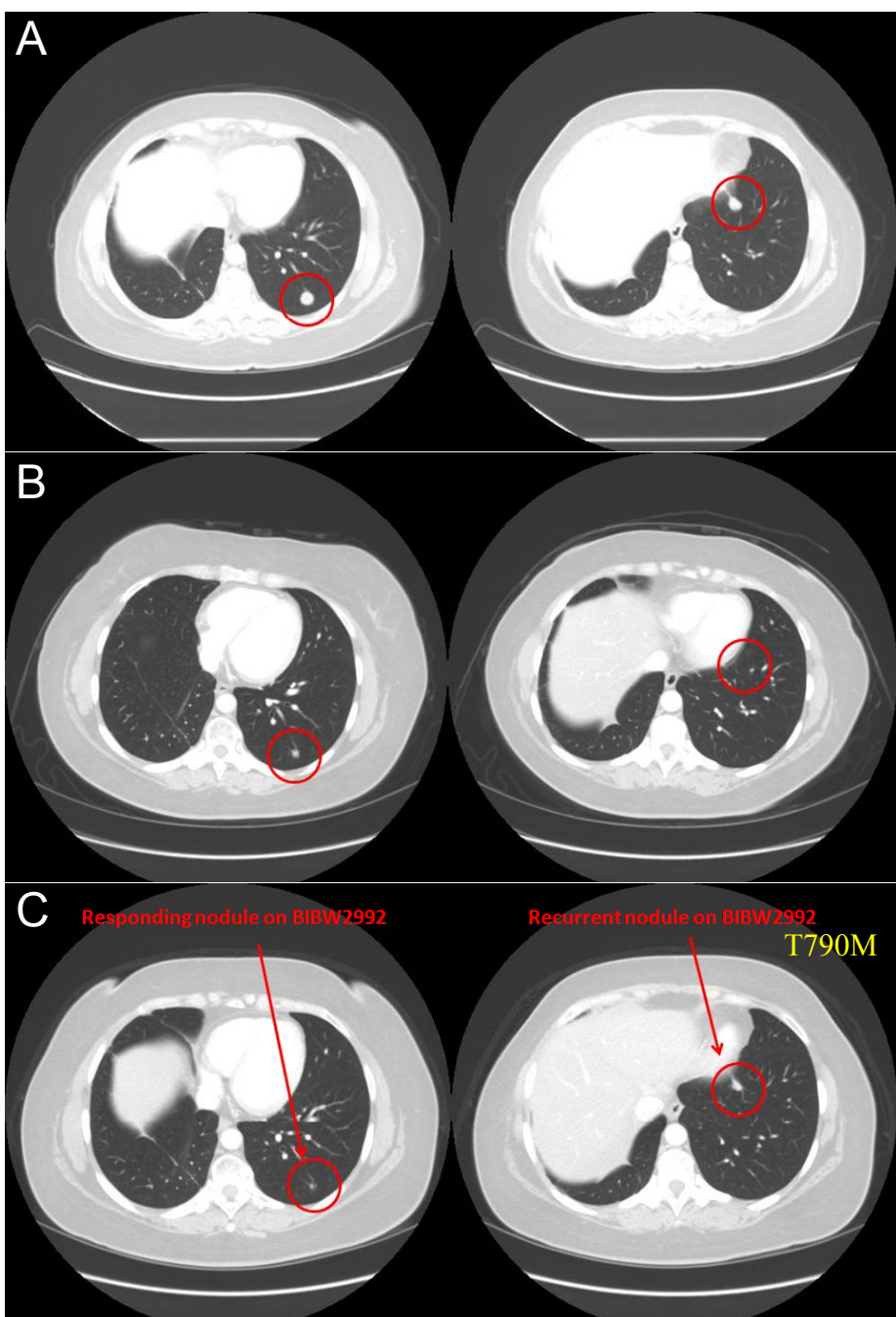


Fig.4