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Intermittent exposure to EGFR tyrosine kinase inhibitors selects less EGFR T790M mutant clones than continuous exposure in lung cancer cell lines

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Abstract:	Background: Drug-resistant cell lines are essential tools for investigating the mechanisms of resistance to molecular-targeted anti-cancer drugs. However, little is known about how to establish clinically relevant drug-resistant cell lines. Our study examined the impact of a drug-free period on the establishment of a cell line with clinically relevant resistance to molecular-targeted drugs. Methods: We used PC-9 cells, a lung cancer cell line carrying EGFR mutation, because this is a validated target for EGFR tyrosine kinase inhibitors (TKI). PC-9 cells were intermittently or continuously exposed to increasing concentrations of gefitinib (0.01 μM to 1.0 μM) and the emergence of the most common acquired resistance mutation in EGFR, T790M, was determined. Results: T790M was detected at a 25-fold lower drug concentration in cells continuously exposed to gefitinib (PC-9/GRc) than in cells intermittently exposed to gefitinib (PC-9/GRi) (0.04 μM vs 1.0 μM, respectively). The mutation frequencies at those drug concentrations were 19.8% and 8.0% in PC-9/GRc and PC-9/GRi cells, respectively. After drug-free culture for 8 weeks, resistance to gefitinib decreased in the PC-9/GRi cells but not in the PC-9/GRc cells. In the PC-9/GRc cells, the frequency of the T790M mutation was consistently about 20% from 0.04 μM to 1.0 μM of gefitinib. In the PC-9/GRc cells, the T790M mutation was detected in all single-cell clones, at frequencies ranging from 7.0% to 37.0%, with a median of 19.5% (95% confidence interval, 17.3%-20.9%). Conclusion: Intermittent exposure to EGFR-TKIs reduces the emergence of the EGFR T790M mutation in a lung cancer cell line.
Keywords:	Drug resistance: Drug sensitivity: EGFR tyrosine kinase inhibitor; EGFR mutation: Non−small−cell lung cancer: Sequencing : Cell line

Intermittent exposure to EGFR tyrosine kinase inhibitors selects less EGFR T790M mutant clones than continuous exposure in lung cancer cell lines

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CONFLICT OF INTEREST DISCLOSURES

The authors indicated no potential conflicts of interest.

Abstract

Background: Drug-resistant cell lines are essential tools for investigating the mechanisms of resistance to molecular-targeted anti-cancer drugs. However, little is known about how to establish clinically relevant drug-resistant cell lines. Our study examined the impact of a drug-free period on the establishment of a cell line with clinically relevant resistance to molecular-targeted drugs.

Methods: We used PC-9 cells, a lung cancer cell line carrying *EGFR* mutation, because this is a validated target for EGFR tyrosine kinase inhibitors (TKI). PC-9 cells were intermittently or continuously exposed to increasing concentrations of gefitinib (0.01 μM to 1.0 μM) and the emergence of the most common acquired resistance mutation in EGFR, T790M, was determined.

Results: T790M was detected at a 25-fold lower drug concentration in cells continuously exposed to gefitinib (PC-9/GRc) than in cells intermittently exposed to gefitinib (PC-9/GRi) (0.04 μM vs 1.0 μM, respectively). The mutation frequencies at those drug concentrations were 19.8% and 8.0% in PC-9/GRc and PC-9/GRi cells, respectively. After drug-free culture for 8 weeks, resistance to gefitinib decreased in the PC-9/GRi cells but not in the PC-9/GRc cells. In the PC-9/GRc cells, the frequency of the T790M mutation was consistently about 20% from 0.04 μM to 1.0 μM of gefitinib. In the PC-9/GRc cells, the T790M mutation was detected in all single-cell clones, at frequencies ranging from 7.0% to 37.0%, with a median of 19.5% (95% confidence interval, 17.3%–20.9%).

Conclusion: Intermittent exposure to EGFR-TKIs reduces the emergence of the EGFR T790M mutation in a lung cancer cell line.

Introduction

Recent advances in identifying and targeting driving gene alterations has allowed innovative treatments for human cancer to be developed, particularly molecular-targeted anticancer therapies. However, the emergence of drug resistance has become an important issue even in the era of molecular-targeted therapies. If we could determine the genetic alterations that drive resistance to molecular-targeted drugs, suppressing these resistance targets could overcome drug resistance in cancer patients. Cancer tissue samples and established cell lines are conventionally used to investigate the mechanisms involved in drug resistance. However, because collecting samples from patients is difficult, cell lines are usually used in experiments to examine the mechanisms underlying acquired resistance to molecular-targeted drugs. The *in vitro* establishment of drug-resistant cell lines is often laborious, and may take from several months to several years. The clinical relevance of artificially developed cell lines also remains controversial, and the most appropriate strategy for establishing drug-resistant cell lines is unclear.

In this study, we examined the impact of a drug-free culture period when establishing a cell line with clinically relevant resistance to a molecular-targeted drug, because the characteristics and strength of the acquired resistance in an established cell line might be influenced by a drug-free interval. Therefore, in this study, a cancer cell line was intermittently or continuously exposed to a molecular-targeted drug and the phenotypes and genotypes of the resistant cells were determined. We used a lung cancer cell line carrying a mutation in the epidermal growth factor receptor gene (*EGFR*) because this is a validated target for EGFR tyrosine kinase inhibitors (EGFR-TKIs)[1]. EGFR-TKIs were the first molecular-targeted drugs to change the

chemotherapeutic approach to lung cancer. These drugs are particularly effective in lung cancers with activating *EGFR* mutations such as exon 19 deletion and exon 21 L858R mutation[1]. However, most cancers that initially respond to EGFR-TKIs eventually acquire drug resistance. Several mechanisms are responsible for acquired resistance to EGFR-TKIs, and the most common is the emergence of the T790M mutation in exon 20 of *EGFR*[2]. This resistance mutation was detected in approximately 60% of rebiopsy samples obtained from patients with acquired resistance to EGFR-TKIs[2]. Furthermore, a preclinical study using established drug-resistant cell lines revealed the molecular mechanism of resistance induced by the T790M in EGFR[3]. Therefore, we measured the frequency of EGFR T790M in *EGFR*-mutant lung cancer cell lines resistant to EGFR-TKIs, which were established using two different drug-treatment regimens, in order to determine the relevance of these regimens to the emergence of clinically relevant drug resistance.

Materials and Methods

Cell line and reagents

PC-9 cells, a human lung cancer cell line carrying a deletion in exon 19 (DelE746A750) of *EGFR*, were purchased from RIKEN BioResource Center Cell Bank (Ibaraki, Japan). The cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. An EGFR-TKI, gefitinib (Iressa®), was purchased from LC Laboratories (Woburn, MA, USA).

Establishment of gefitinib-resistant cell lines

The gefitinib-resistant cell line (PC-9/GR) 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 1A). 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. An aliquot of cells was stored before each increase in the dose of gefitinib.

Cell viability assay

The cells were cultured in gefitinib-free medium for ≥ 1 week before testing. 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.

Direct sequencing

Genomic DNA was extracted from the cells with a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. 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′-CCATGAGTACGTATTTTGAAAC-TC-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.

Mass spectrometry (MS) assay

The EGFR T790M mutation was detected in the cells with matrix-assisted laser

desorption/ionization time-of-flight MS using a standard protocol, on the MassARRAY System (Sequenom, San Diego, CA, USA). The mutant signal frequency was calculated as follows: mutant signal frequency (%) = (mutant peak height)/(mutant peak height + wild-type peak height) × 100.

Single-cell clone assay

Individual clones were established from the resistant cell lines. Briefly, 4×10^3 drug-resistant cells were serially diluted and then incubated in 96-well plates. Each well was checked to identify those that contained a single colony. These colonies were picked from the wells and subcultured in larger vessels.

Results

Generation of two drug-resistant cell lines

With continuous exposure to gefitinib (PC-9/GRc cells), drug resistance was observed after 42 weeks, whereas with intermittent exposure (PC-9/GRi cells), drug resistance was observed after 18 weeks. The gross morphologies and growth rates of both resistant cell lines were similar (Figure 1B). Both cell lines also displayed similar sensitivities to gefitinib (gefitinib IC_{50}, 17.8 μM in PC-9/GRc and 15.8 μM in PC-9/GRi) (Figure 1C).

Long-term stability of gefitinib resistance

To assess the long-term stability of gefitinib resistance, the sensitivity of both cell lines to gefitinib was measured after drug-free culture for 8 weeks. The sensitivity of the PC-9/GRc cells to gefitinib was not significantly different before and after drug-free culture (gefitinib IC_{50}, 17.0 μM and 13.5 μM before and after drug-free culture, respectively) (Figure 2A). By contrast, the sensitivity of the PC-9/GRi cells was significantly higher after drug-free culture compared with their sensitivity before drug-free culture lines (gefitinib IC_{50}, 15.9 μM and 1.04 μM before and after drug-free culture, respectively) (Figure 2B).

T790M frequency

We next compared the frequency of the EGFR T790M mutation in the two drug-resistant cell lines. The MS assay was performed in both cell lines established at serially increasing concentrations of gefitinib (Figure 3). The lowest drug concentration at which T790M was detected was 0.04 μM in PC-9/GRc cells and 1.0

μM in PC-9/GRi cells. The mutant allele frequency at the lowest drug concentration at which T790M was detected was 19.8% in PC-9/GRc cells and 8.0% in PC-9/GRi cells.

In PC-9/GRc cells, the allele frequency of T790M was approximately 20% at the lowest drug concentration at which T790M was detected and more, even at the highest drug concentration. The frequency of the T790M mutation in these cells did not increase as the drug concentration increased. These results are consistent with the results obtained with direct sequencing (Figure 4). Although the height of the mutant chromatogram peak was low, the peak corresponding to T790M was detected at each concentration from 0.04 μM to 1.0 μM in PC-9/GRc cells, but was not detected in the PC-9/GRi cells. The direct sequencing chromatogram also showed that the height of the T790M peak in the PC-9/GRc cells did not increase as the drug concentration increased.

Intra-cell-line T790M heterogeneity

We examined whether there was intra-cell-line heterogeneity in the frequency of the EGFR T790M mutation in the PC-9/GRc cells. Overall, 54 single-cell clones were isolated from PC-9/GRc cells established with continuous exposure to 1.0 μM gefitinib. The MS assay for T790M was performed for each single-cell clone. T790M was detected in all single-cell clones, but the allele frequencies ranged considerably, from 7.0% to 37.0%, with a median of 19.5% (95% confidence interval 17.3%–20.9%; Figure 5). The frequency of T790M in PC-9/GRc clone 51 was substantially lower than that in PC-9/GRc clone 49, so these two representative single-cell clones displayed the heterogeneity of T790M. This heterogeneity was also detected with direct sequencing (Figure 6).

Discussion

Our study has demonstrated that intermittent drug exposure led to the establishment of cells with less stable drug resistance compared with that of cells exposed continuously to the drug gefitinib. Intermittent drug exposure was less effectively induced the emergence of the EGFR T790M mutation than continuous drug exposure. Using a standard direct sequencing method with a detection limit of about 20%, we could not detect *EGFR* T790M in the PC-9/GRi cells, even when they were most resistant to gefitinib. These findings are consistent with those of another study in which the researchers used intermittent drug exposure to establish a lung cancer cell line resistant to an EGFR-TKI[4]. Rho *et al*. generated resistant PC-9 cells by exposing them to gefitinib or erlotinib for 48 h, maintaining them in drug-free medium, and then re-exposing them to increasing concentrations of the drug to a final concentration of 1.0 μM. Like us, they were unable to detect T790M in the drug-resistant cell lines with direct sequencing, although when they used the more sensitive pyrosequencing method, the mutation frequency was 13%~14%. These results suggest that the drug-sensitive clones can be restored when the drug selection pressure is removed, thus supressing the expansion of drug-resistant clones. Taken together, these findings indicate that continuous drug exposure offers an advantage over intermittent exposure because when cell lines are created, it selects better minor drug-resistant clones that are resistant to molecular-targeted drugs.

Several other studies support our finding that continuous exposure to a molecular-targeted drug selects better drug-resistant tumor cells than intermittent exposure[5,6]. For example, using a mathematical cancer model and *EGFR*-mutant

lung cancer cell lines, Chmielecki *et al.* showed that a high–dose pulse dosing combined with a continuous low dosing of EGFR-TKI delayed the emergence of T790M-mediated resistance compared with its emergence in cells treated with a continuous standard dosing[5]. This research group has implemented a phase I clinical trial based on these preclinical data. The results of their studies should help us improve the clinical efficacy of EGFR-TKIs in the treatment of *EGFR*-mutant lung cancers by delaying the emergence of drug resistance. Thakur *et al*. reported that drug-resistant cells showed continuous dependence on a B-RAF inhibitor in *BRAF*-mutated melanoma cell lines and that an intermittent dosing strategy delayed the onset of drug resistance in a xenograft tumor model[6]. Based on these preclinical data, a clinical trial has been commenced to compare the efficacy of intermittent dosing and continuous dosing with a B-RAF inhibitor and a MEK inhibitor in patients with *BRAF*-mutant melanoma.

Interestingly, there is an apparent drug concentration at which the expansion of drug-resistant clones plateaus, because the frequency of T790M remained constant once the drug concentration exceeded this value. This indicates that the strength of resistance does not correlate positively with the frequency of the resistance mutation. Unfortunately, we could not determine the clinical relevance of these results because there are no clinical data on the frequency of the EGFR T790M mutation in drug-resistant tumor tissues. However, if we knew the threshold concentration at which drug resistance is expected to develop, we could save time and labor in producing cell lines with resistance to molecular-targeted drug that mimic the molecular characteristics of human tumors. In addition, we observed the intra-cell-line heterogeneity in the mutant allele frequency. Therefore, it is necessary to consider

clonal diversity in the phenotypes and genotypes of single-cell drug-resistant clones when they are used to examine the mechanisms of drug resistance.

In conclusion, cancer cell lines are an important tool in studying the mechanisms underlying drug resistance in human cancer. Our findings indicate that the mechanisms responsible for the resistant phenotype of cell lines can vary according to the conditions used to establish the cells, especially the use of intermittent or continuous drug exposure regimens. Compared with intermittent drug exposure, continuous drug exposure might select better minor resistant clones when creating cell lines resistant to molecular-targeted drugs.

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Figure legends

Figure 1. Characterization of two cell lines established with continuous or intermittent exposure to gefitinib. (A) Schematic summary of two drug treatment methods (B) Both cell lines grew at similar rates over 2 weeks. (C) Cell viability assay was conducted after 72 h incubation on gefitinib. Both cell lines were resistant to gefitinib at similar concentrations (gefitinib IC_{50}, 17.8±1.2 μM in PC-9/GRc and 15.8±1.3 μM in PC-9/GRi). The graphs show the mean values of triplicate experiments and the error bars represent the standard deviations.

Figure 2. Long-term stability of gefitinib resistance in two cell lines established with continuous or intermittent exposure to gefitinib. PC-9/GRc and PC-9/GRi cells were cultured in gefitinib-free medium for 8 weeks and their drug sensitivity was measured with an MTS cell proliferation assay after 72 h incubation on gefitinib. (A) The sensitivity of PC-9/GRc cells to gefitinib was not significantly increased by drug-free culture (gefitinib IC_{50}, 17.0±1.2 μM and 13.5±1.2 μM before and after drug-free culture, respectively). (B) The sensitivity of PC-9/GRi cells to gefitinib increased significantly after drug-free culture (gefitinib IC_{50}, 15.9±1.2 μM and 1.04±2.1 μM before and after drug-free culture, respectively). The graphs show the mean values of triplicate experiments and the error bars represent the standard deviations.

Figure 3. Differences in the frequency of the EGFR T790M mutation in the two cell lines established with continuous or intermittent exposure to gefitinib. The frequency of T790M was measured semiquantitatively in both cell lines at each concentration of gefitinib with a mass spectrometry assay.

Figure 4. Direct sequencing chromatograms of *EGFR* exon 20 revealed the presence of T790M (*ACG→ATG) in PC-9/GRc cells at gefitinib concentrations ranging from 0.04 μM to 1.0 μM, but not in PC-9/GRi cells.

Figure 5. Distribution of the *EGFR* T790M mutant allele frequency in 54 single-cell clones isolated from PC-9/GRc cells exposed to 1.0 μM gefitinib. The median frequency was 19.5% (95% confidence interval 17.3%–20.9%).

Figure 6. Direct sequencing chromatograms of *EGFR* exon 20 showed a difference in the peak at the site of the T790M mutation between two different single-cell clones derived from PC-9/GRc cells.

Figure

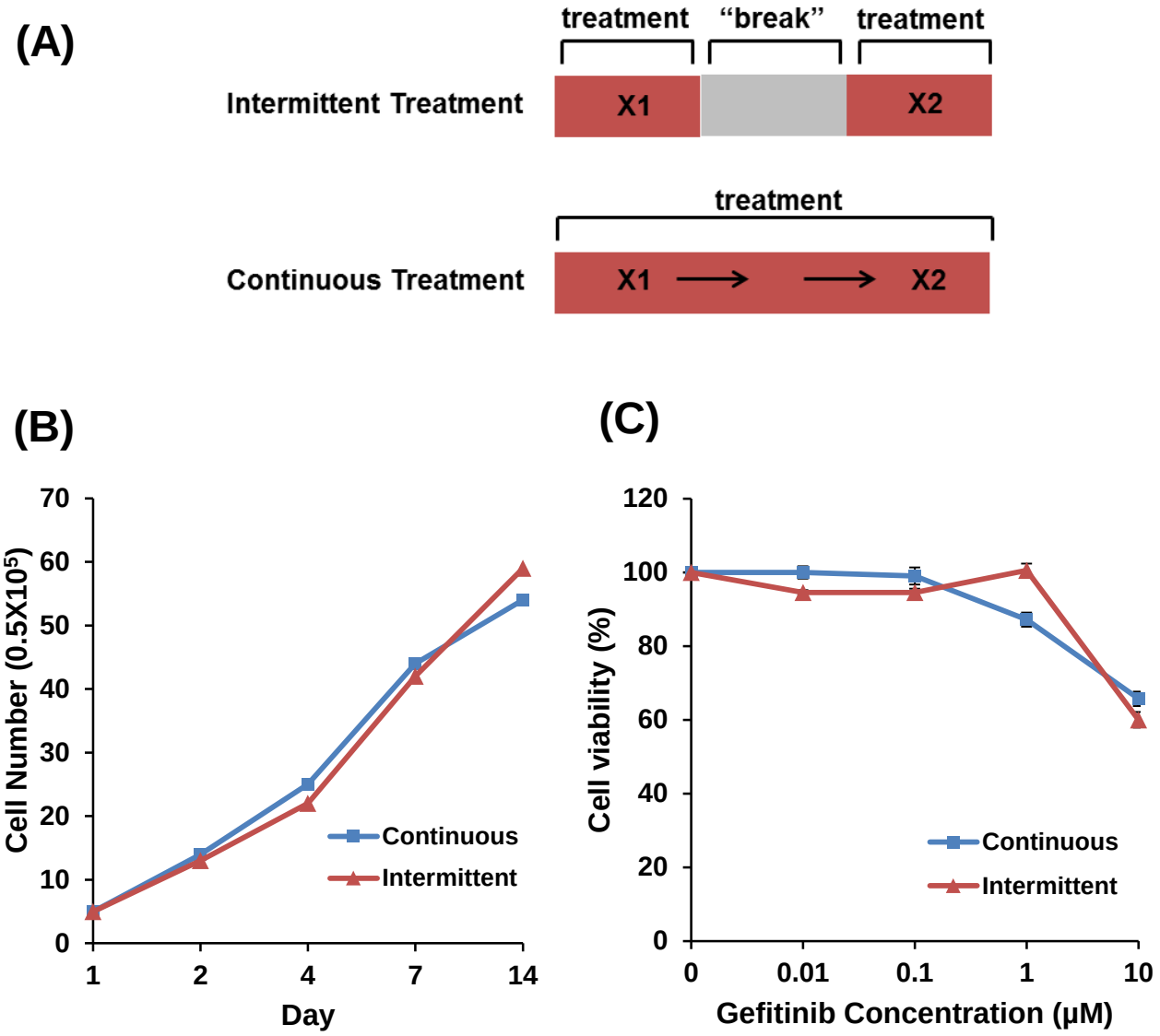


Figure 1.

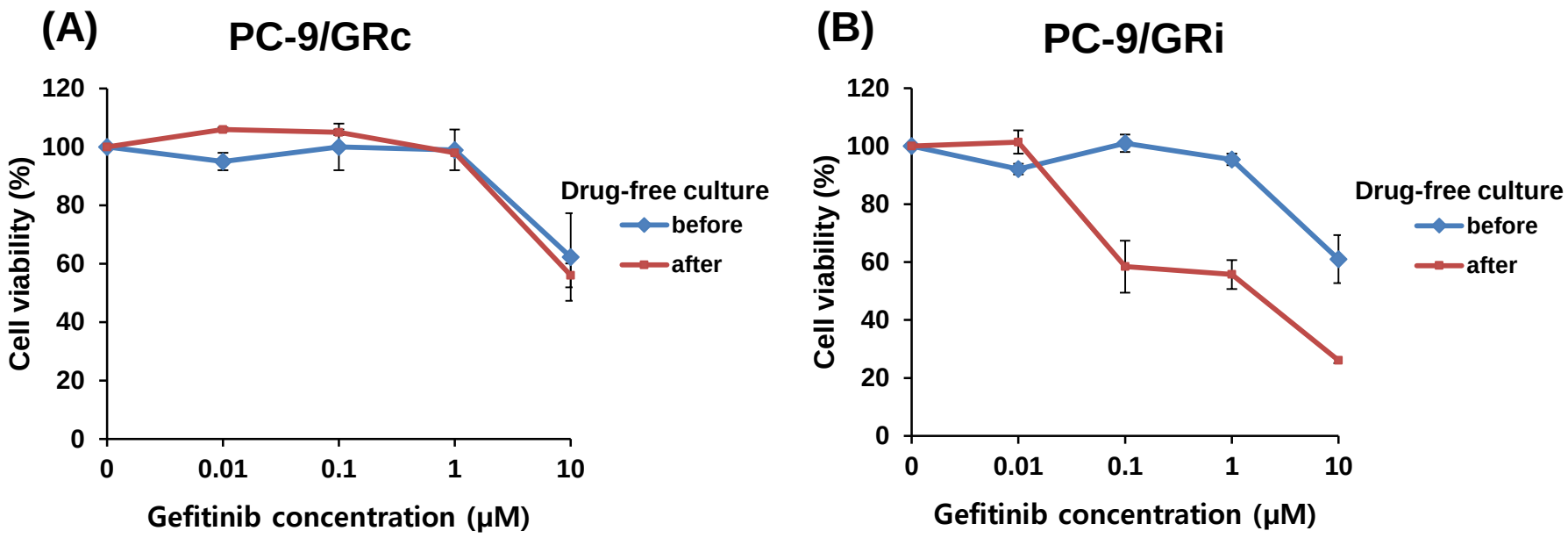
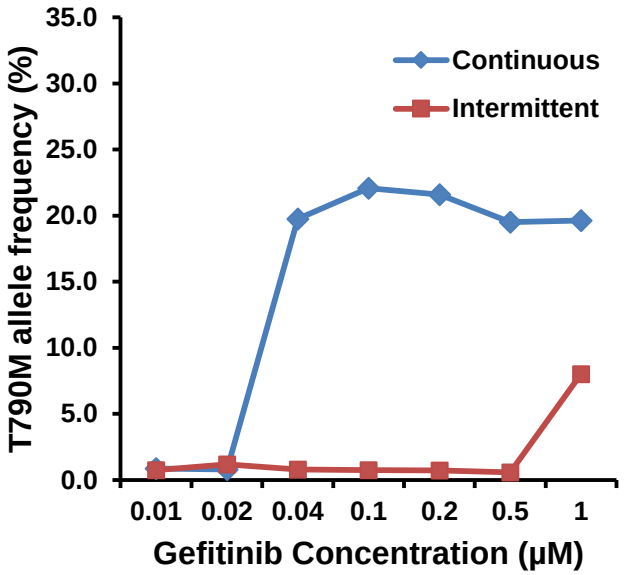

(A)
PC-9/GRc
Cell viability (%)
120
100
80
60
40
20
0
0
0.01
0.1
1
10
Gefitinib concentration (μM)
Drug-free culture
before
after
(B)
PC-9/GRi
Cell viability (%)
120
100
80
60
40
20
0
0
0.01
0.1
1
10
Gefitinib concentration (μM)
Drug-free culture
before
after

Figure 2.



Drug Conc. (μM)	T790M frequency (%)	
	Continuous treatment	Intermittent treatment
0.01	0.9	0.8
0.02	0.8	1.2
0.04	19.8	0.8
0.1	22.1	0.8
0.2	21.6	0.7
0.5	19.5	0.6
1.0	19.6	8.0

Figure 3.

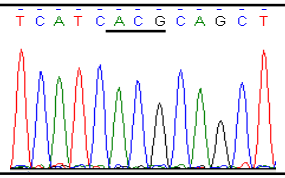
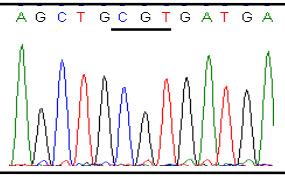
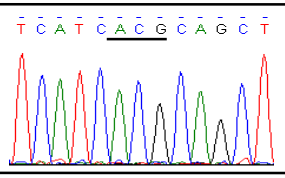
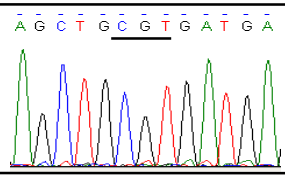
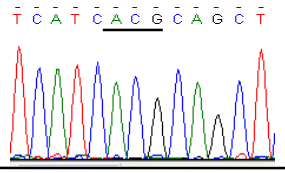
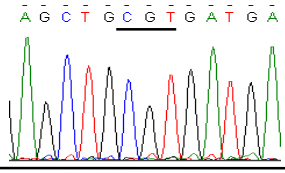
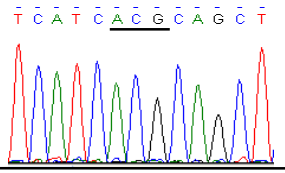
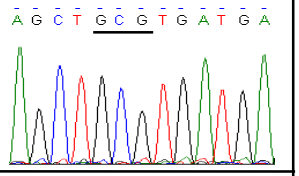
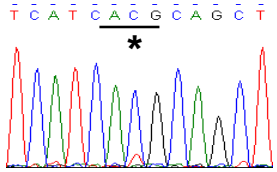
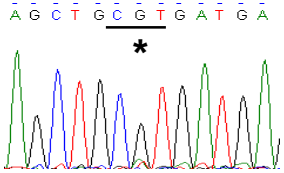
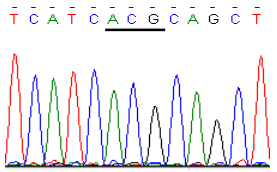
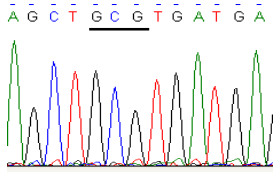
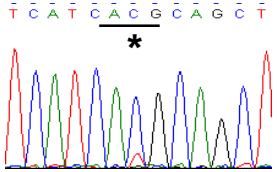
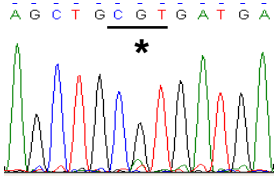
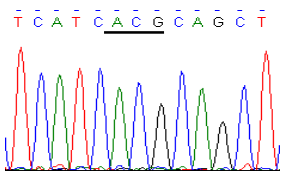
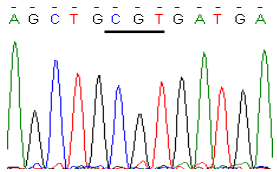
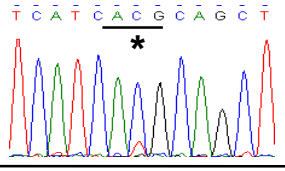
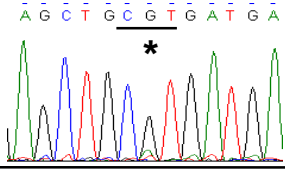
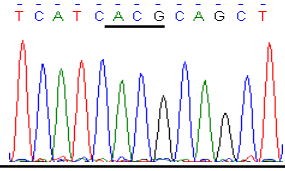
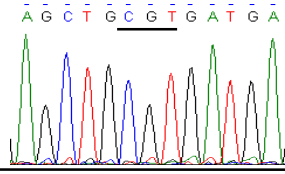
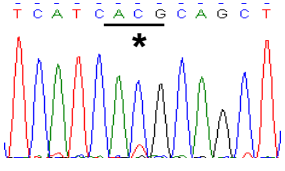
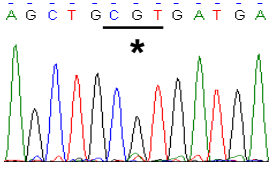
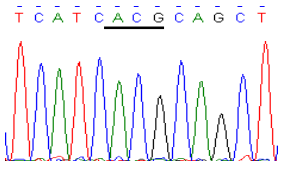
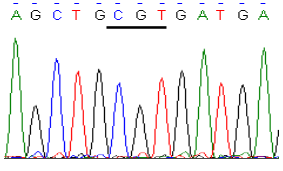
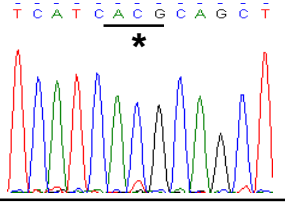
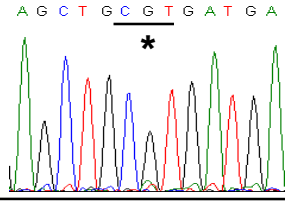
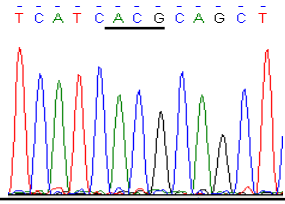
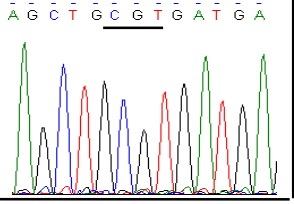
Drug Conc.	Continuous treatment		Intermittent treatment	
	Forward	Reverse	Forward	Reverse
0.01μM				
0.02μM				
0.04μM				
0.1μM				
0.2μM				
0.5μM				
1.0μM				

Figure 4.

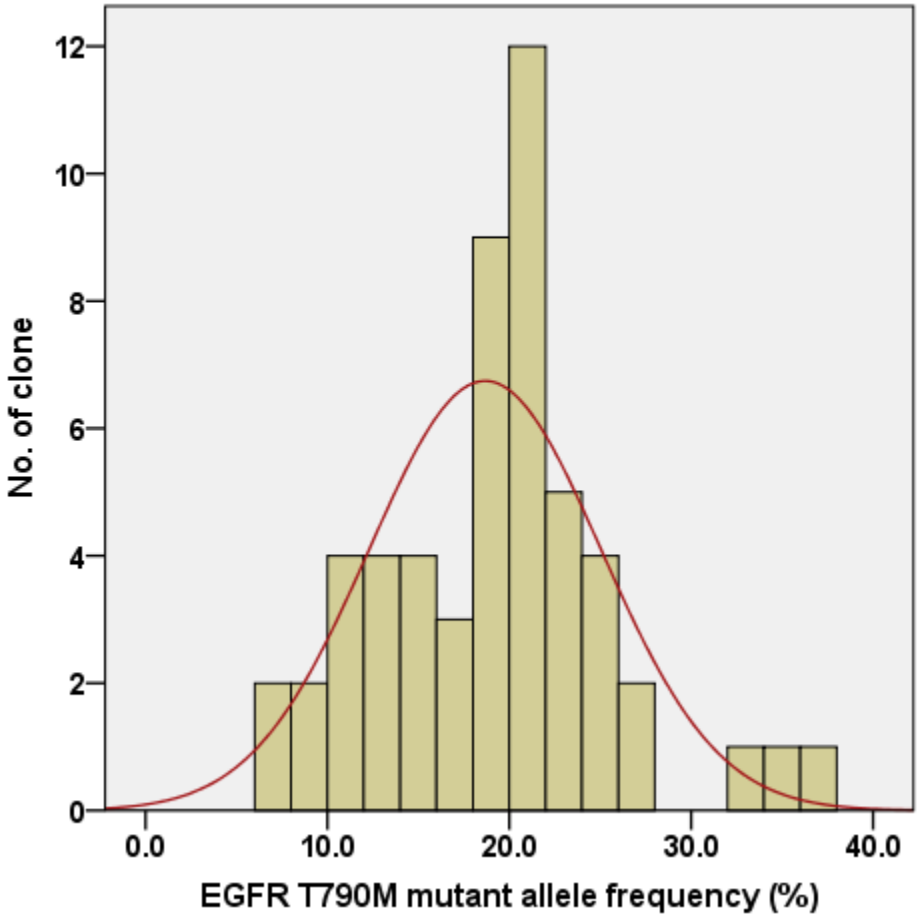

No. of clone
12
10
8
6
4
2
0
0.0
10.0
20.0
30.0
40.0
EGFR T790M mutant allele frequency (%)

Figure 5.

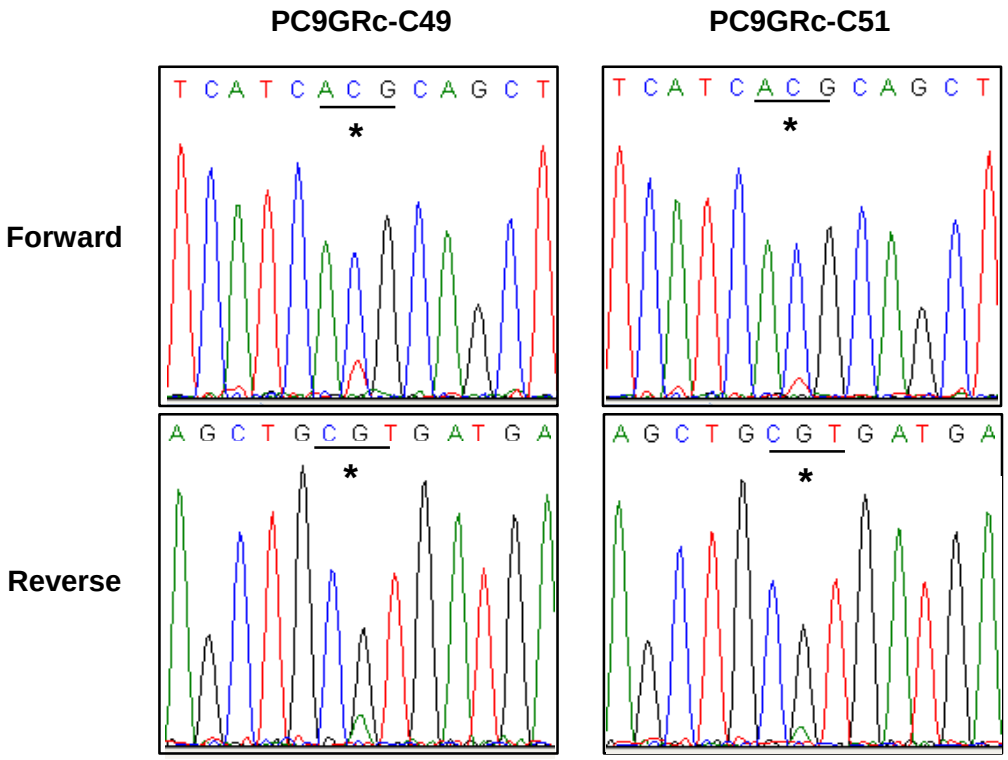

PC9GRc-C49
PC9GRc-C51
Forward
Reverse
T C A T C A C G C A G C T
A G C T G C G T G A T G A
*
EGFR exon20 T790M mutation

Figure 6.