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TITLE:

Establishment and Characterization of Three Afatinib-Resistant Lung Adenocarcinoma PC-9 Cell Lines Developed with Increasing Doses of Afatinib

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KEYWORDS:

stepwise dose escalation, drug Resistance, EGFR-activating mutations, EGFR-TKI, afatinib, lung cancer

SHORT ABSTRACT:

A method for establishing afatinib-resistance cell lines from lung adenocarcinoma PC-9 cells was developed, and resistant cells were characterized. The resistant cells can be used to investigate epidermal growth factor receptor tyrosine kinase inhibitor-resistance mechanisms, applicable for patients with non-small cell lung cancer.

LONG ABSTRACT:

Acquired resistance to molecular target inhibitors is a severe problem in cancer therapy. Lung cancer remains the leading cause of cancer-related death in most countries. The discovery of “oncogenic driver mutations,” such as epidermal growth factor receptor (*EGFR*)-activating mutations, and subsequent development of molecular targeted agents of EGFR tyrosine kinase inhibitors (TKIs) (gefitinib, erlotinib, afatinib, dacomitinib, and osimertinib) have dramatically altered lung cancer treatment in recent decades. However, these drugs are still not effective in patients with non-small cell lung cancer (NSCLC) carrying *EGFR*-activating mutations. Following acquired resistance, the systemic progression of NSCLC remains a significant obstacle in treating patients with EGFR mutation-positive NSCLC. Here, we present a stepwise dose escalation method for establishing three independent acquired afatinib-resistant cell lines from NSCLC PC-9 cells harboring EGFR-activating mutations of 15-base pair deletions in EGFR exon 19. Methods for characterizing the three independent afatinib-resistance cell lines are briefly presented. The acquired resistance mechanisms to EGFR TKIs are heterogeneous. Therefore, multiple cell lines with acquired resistance to EGFR-TKIs must

be examined. Ten to twelve months are required to obtain cell lines with acquired resistance using this stepwise dose escalation approach. The discovery of novel acquired resistance mechanisms will contribute to the development of more effective and safe therapeutic strategies.

INTRODUCTION:

Five tyrosine kinase inhibitors, targeting epidermal growth factor receptor (EGFR), including gefitinib, erlotinib, afatinib, dacomitinib, and osimertinib are currently available for treating patients with EGFR mutation-positive non-small cell lung cancer (NSCLC). Over the past decade, therapies for such patients have undergone dramatic development with the discovery of new potential EGFR-TKIs. Among patients with lung adenocarcinoma, somatic mutations in *EGFR* are identified in approximately 50% of Asian and 15% of Caucasian patients[1]. The most common mutations in EGFR are an L858R point mutation in EGFR exon 21 and 15-base pair (bp) deletions in EGFR exon 19[2]. In EGFR mutation-positive patients with NSCLC, EGFR-TKIs improve the response rates and clinical outcomes compared to the previous standard of platinum doublet chemotherapy[3].

Gefitinib and erlotinib were the first approved small molecule inhibitors and are generally referred to as first-generation EGFR TKIs. These EGFR TKIs block tyrosine kinase activity by competing with ATP and reversibly binding to ATP binding sites[4]. Afatinib is a second-generation EGFR TKI that irreversibly and covalently binds to the tyrosine kinase domain of EGFR and is characterized as a pan-human EGFR family inhibitor[5].

Despite the dramatical benefit of these therapies in patients with NSCLC, acquired resistance is inevitable. The most common resistance mechanism against first- and second-generation EGFR TKIs is the emergence of the T790M mutation in EGFR exon 20, which is present in 50–70% of tumor samples[6-8]. Other resistance mechanisms include bypass signals (to MET, IGF1R, and HER2), transformation to small cell lung cancer, and induction of epithelial-to-mesenchymal transition, which occur pre-clinically and clinically[9]. The resistance mechanisms to EGFR TKIs are heterogeneous. By identifying novel resistance mechanisms in preclinical studies, it may be possible to develop novel therapeutics to overcome resistance. Optimal sequence therapies that maximize the clinical benefit to patients must consider the resistance mechanisms and therapeutic target.

It is imperative to choose the right parental cell line, as it is the basis of all the subsequent experiments. The selection strategies begin with clinical relevance; it is necessary to choose a chemotherapy and radiation naïve cell line. Previous chemotherapeutic and/or radiative treatment may induce the alteration of resistance pathways and changes of the expression of drug resistance markers. In this study, PC-9 cells, carrying 15-bp deletions in EGFR exon 19, are employed for the establishment of acquired resistance to afatinib. This cell line was derived from a Japanese NSCLC patient, who did not receive prior chemotherapy and radiation.

Because afatinib is administered orally on a daily basis, continuous in vitro treatment, where the cells are cultured constantly in the presence of afatinib would be clinically relevant. The dose of drugs used in the various steps of the experiment must be optimized for the parental

cell line selected. A cytotoxicity assay can be used for determining a suitable drug range, which should be comparable to the pharmacokinetic information of the drug.

Throughout the selection process, the whole population of cells is maintained as a single group; cloning or other separation methods are not used. The cells are first continuously exposed to a low level of the drug. Subsequently, after the cells adapt to grow in the presence of the drug, the dose of the drug is slowly increased to the final optimal dose of drug[10,11]. Alternatively, a pulse drug-administration or mutagenesis can be used for selecting resistance cells, which are also performed prior to drug treatment [12,13]. Unfortunately, cases where drug resistance fails to develop are generally not reported. The selection strategies are developed with the aim of trying to mimic the conditions of cancer patients for rebuilding clinically relevant resistance. Sometimes, to identify molecular changes associated with mechanisms of drug resistance, a high drug concentration is used. This model becomes less clinically relevant.

Here, we describe a method for establishing three independent afatinib-resistant cell lines from PC-9 cells harboring 15-bp deletions in EGFR exon 19 as well as the initial characterization of the afatinib-resistant cell lines.

PROTOCOL:

1. Establishment of three independent afatinib-resistant PC-9 cell lines

1.1. Determination of the initial afatinib exposure concentration for PC-9 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay

1.1.1. Culture PC-9 cells in growth medium containing fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL) in a cell-culture treated 10-cm dish in a 5% CO_2 incubator at 37 °C.

1.1.2. Resuspend PC-9 cells at 4 × 10^4 cells/mL in growth medium and then seed at 50 µL/well in a 96-well microplate. The final concentration of cells is 2.0 × 10^3 cells/50 µL/well. Incubate overnight in a 5% CO_2 incubator at 37°C.

1.1.3. Add 50 µL of afatinib solution at different concentrations: 0, 0.002, 0.006, 0.02, 0.06, 0.2, 0.6, 2, 6, and 20 µM to the wells containing the growth medium (50 µL). The final volume and concentrations of afatinib are 100 µL and 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µM, respectively.

1.1.4. Incubate the 96-well plate for 96 h in a 5% CO_2 incubator at 37 °C.

1.1.5. Add 15 µL of the dye solution (see **Table of Materials**) to each well and incubate for 4 h in a 5% CO_2 incubator at 37 °C, and then add 100 µL of solubilization/stop-solution (see **Table of Materials**) to each well and incubate overnight in a 5% CO_2 incubator at 37 °C.

1.1.6. Measure the optical density at 570 nm (OD_{570}) using a microplate reader (see **Table of Materials**). Prepare 6–12 replicates and repeat the experiments at least three times.

1.1.7. Use statistical software (see **Table of Materials**) to graphically plot these data as a semi-log graph and calculate the IC_{50} value, which is the drug concentration that reduces response to 50% of its maximum (see **Table of Materials**).

1.2. Continuous exposure of PC-9 cells to the irreversible EGFR-TKI, afatinib, by stepwise dose escalation in three independent 10-cm dishes

1.2.1. Culture PC-9 cells in p100 dishes containing 10 mL of growth medium. When the PC-9 cells reach the sub-confluent stage, transfer 1 mL of cell suspension into three new p100 dishes, with 9 mL of growth medium. The 1:10 diluted PC-9 cells become sub-confluent in 3-4 days, with a cell number of approximately 4-5 × 10^5 cells/mL.

1.2.2. On the next day, add 1/10 of the IC_{50} value of afatinib into each of the three p100 dishes.

NOTE: Afatinib is reconstituted in DMSO at stock concentrations of 1 µM, 10 µM, 100 µM, 1 mM, and 5 mM. 1 to 10 µL of afatinib-solution is added into 10 mL of growth medium in the culture, as per the required final concentrations.

1.2.3. When the cells in the afatinib-containing p100 dishes become sub-confluent, mix well by aspiration with a 1-mL pipette and add 1 mL of the cell suspension to 9 mL of fresh growth medium in a new p100 dish. Next, add 10–20% higher concentrations of afatinib to the new culture.

1.2.4. Increase the afatinib concentration of 0.1 nM to 1 µM in the medium by the stepwise dose escalation with the afatinib concentration increased by 10–20% at each step over the period of 10-12 months.

NOTE: When the afatinib concentration approaches the IC_{50} value, cell growth becomes quite slow. If the cells are split 1:9, they may not grow, as these cells are killed by higher concentrations of afatinib. Therefore, at higher afanitib concentrations, the cells can be split at a ratio of 1:2. The most resistant cells were grown in afatinib-contained medium for 3–14 days, and the medium was not changed until the resistant cells needed to be passaged.

1.2.5. Culture the afatinib-resistant cells for 2–3 months in 1 µM afatinib-containing growth medium. At an afatinib concentration of 1 µM, 10–12 months are required for developing resistance to afatinib in this model. Perform the MTT assay to confirm that the cells are resistant to afatinib. The three independently established afatinib resistance cell lines were named AFR1, AFR2, and AFR3.

2. Characterization of three independent afatinib-resistant cells

2.1. Determination of the growth curve of parental PC-9 cells and establishment of afatinib-resistant cells

2.1.1. Culture the PC-9, AFR1, AFR2, and AFR3 cells in growth medium in a 5% CO_2 incubator at 37 °C.

2.1.2. Resuspend the cells at 5 × 10^3 cells/mL with growth medium, and seed 100 µL/well into a 96-well microplate, such that the final concentration of cells is 500 cells/100 µL/well.

NOTE: The MTT assay is performed to measure the OD_{570} values at 0, 1, 2, 3, 5, and 7 days. Six 96-well microplates are required for each day.

2.1.3. Perform the MTT assay every 24 h and then on days 0, 1, 2, 3, 5, and 7. Measure the OD_{570} values and prepare 6–12 replicates; repeat the experiments at least three times, and graphically plot the results using a statistical software (see **Table of Materials**).

2.2. Identification of the genomic DNA alterations in EGFR by real-time PCR

NOTE: Afatinib is a small molecule inhibitor that targets EGFR tyrosine kinase. The EGFR expression status is determined at the DNA and protein levels.

2.2.1. Genomic DNA is isolated using a DNA purification kit (see **Table of Materials**) following the manufacturer's instructions. Measure the concentration of the isolated genomic DNA with a spectrophotometer (see **Table of Materials**) and adjust all genomic DNA samples to 25 ng/ µL.

2.2.2. Amplify 50 ng of genomic DNA, which is equivalent to 2 µL of the 25 ng/µL stocks, using a SYBR Green master mix (see **Table of Materials**) and analyze the results using a fluorescence-based RT-PCR-detection system (see **Table of Materials**).

NOTE: PCR cycling conditions began with an initial denaturation step at 95 °C for 20 s, followed by 40 cycles of 95 °C denaturation for 3 s, 60 °C annealing for 30 s. The specific primer sets are as follows: EGFR F: 5'-CAAGGCCATGGAATCTGTCA-3', R: 5'-CTGGAATGAGGTGGAGGAACA-3'. Normalization gene LINE-1 F: 5'-AAAGCCGCTCAACTACATGG-3', R: 5'-TGCTTTGAATGCGTCCCAGAG-3'.

2.3. Evaluation of the effect of protein alterations on the EGFR level by western blot analysis

2.3.1. Treat the cells with afatinib continuously prior to experiments for 24 h. Wash PC-9, AFR1, AFR2, and AFR3 cells twice with PBS and then seed them in growth media without afatinib. Wash PC-9, AFR1, AFR2, and AFR3 cells twice with 5 mL of ice-cold PBS.

2.3.2. Lyse the cells in RIPA buffer containing 0.1% protease cocktail (see **Table of Materials**) and phosphatase inhibitor II and III (see **Table of Materials**) and incubate this solution at 4 °C for 30 min. Centrifuge the lysates for 10 min at 100 × *g* and 4 °C and collect the cleared lysates.

2.3.3. Determine protein concentrations using the bicinchoninic acid assay (see **Table of Materials**), adjust all protein samples to 0.5 or 1 µg/µL using 4x sample buffer (500 mM Tris (PH 6.8), 40% glycerol, 8% SDS, 20% H_2O, 0.02% bromophenol blue) and boil at 96 °C for 5 min. Store these protein samples at -80 °C until western blot analysis is performed.

2.3.4. Separate equal amounts of protein samples, preferably 20–30 µL, by 8% SDS-page and transfer the proteins to a polyvinylidene fluoride (PVDF) membrane.

NOTE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used in the lab for the separation of proteins based on their molecular weight.

2.3.4.1. Clean glass plates with ethanol and assemble the glass plate and spacers. Prepare 8% poly-acrylamide gels containing 1.5 M Tris-HCl, pH 8.8, 40% Bis-acrylamide, 10% SDS, 10% APS, and TEMED. Polymerize for 30 min at room temperature.

2.3.4.2. Subsequently, prepare a stacking gel containing 0.5 M Tris-HCl, pH 6.8, 40% bis-acrylamide, 10% SDS, 10% APS, and TEMED. Add the stacking gel solution, insert the comb, and polymerize the gel for 20-30 min at room temperature.

2.3.4.3. Place the gels in the electrophoresis apparatus and fill the tank with running buffer (0.25 M Tris, 1.92 M glycine, and 1% SDS). Load equal amount of protein samples (20-30 µL) and run the gel at 180 V. Stop electrophoresis once the dye front flows out of the gel, after approximately 60 min.

2.3.4.4. Wash the gel for 1–2 min with TBST and then transfer the proteins on to a PVDF membrane by semi-dry blotting (see **Table of Materials**) for 1.5 h at a constant current of 300 mA.

2.3.5. Block the membranes with 5% of nonfat dry milk (see **Table of Materials**) diluted with TBST solution (see **Table of Materials**) for 1 h at room temperature, and then probe the membranes with anti-EGFR, anti-phospho-EGFR (Y1068), anti-HER2, anti-HER3, anti-MET, and anti-actin antibodies (diluted 1:3000 in TBST) (see **Table of Materials**) at 4 °C overnight.

2.3.6. Wash the membranes with TBST three times for 10 min, and then expose the membranes to the secondary antibody (diluted 1:200 in TBST) for 1–1.5 h at room temperature. Wash the membranes five times with TBST for 10 min at room-temperature, expose them to the ECL solution (see **Table of Materials**), and visualize the signals using films.

2.4. Analysis of EGFR mutations by sequencing

2.4.1. Amplify genomic DNA using specific primers for EGFR exons 19–21. The PCR cycling conditions begin with an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min.

NOTE: The specific primers for EGFR exon 19: F: 5'-GCAATATCAGCCTTAGGTGCGGCTC-3' R: 5'-CATAGAAAGTGAACATTTAGGATGTG-3', exon 20: F: 5'-CCATGAGTACGTATTTTGAAACTC-3', R: 5'-CATATCCCCATGGCAAACTCTTGC-3', and exon 21: F: 5'-ATGAACATGACCCTGAATTCGG-3', R: 5'-GCTCACCCAGAATGTCTGGAGA-3'.

2.4.2. Purify the amplified PCR products using a PCR purification kit (see **Table of Materials**) and sequence the amplicons.

REPRESENTATIVE RESULTS:

The schema for establishing three afatinib-resistance cell lines from PC-9 cells using a stepwise dose-escalation procedure is shown in **Figure 1**. **Figure 2** shows a decrease in cell proliferation of parental PC-9 cells as the concentration of afatinib is increased, indicating that PC-9 cells are sensitive to afatinib exposure. **Figure 3** shows the afatinib-resistance of the three cell lines. None of the three afatinib-resistant cell lines, AFR1, AFR2, and AFR3, showed suppression of cell proliferation under afatinib exposure. **Figure 4** shows the cell-proliferation curves for PC-9, AFR1, AFR2, and AFR3 cells. The three afatinib-resistant cell lines exhibited significantly slower growth than the parental PC-9 cells. **Figure 5** shows the expression levels of EGFR gDNA in PC-9 and the three afatinib-resistant cells, which indicate that afatinib-resistant cells expressed significantly higher levels of EGFR gDNA than the parental PC-9 cells. **Figure 6** shows the protein expression of EGFR in PC-9 and afatinib-resistant cells. At comparable gDNA expression levels, EGFR protein expression was higher in resistant cells than in parental PC-9 cells. **Figure 7** shows that the sequencing results of EGFR exons 19 and 20 in PC-9, AFR1, AFR2, and AFR3 cells. PC-9 cells showed 15-bp deletions in EGFR exon 19 and wild-type EGFR in exon 20. However, AFR1 and AFR2 cells exhibited amplification of wild-type EGFR exon 19. AFR3 cells contained 15-bp deletions in EGFR exon 19 as in PC-9 cells, but the point mutation T790M was observed in EGFR exon 20.

FIGURE AND TABLE LEGENDS:

Figure 1: Schema of the process used to establish three afatinib-resistant cell lines from PC-9. First, PC-9 cells were separated into three p100 dishes and exposed to afatinib at 1/10 of the IC_{50} value. Next, afatinib concentrations in the growth medium were increased by stepwise dose escalation to 1 µM. After 10–12 months, three independent afatinib-resistant cell lines were established and named AFR1, AFR2, and AFR3.

Figure 2: Parental PC-9 cells are sensitive to the irreversible EGFR TKI, afatinib. Cells were seeded into a 96-well microplate at 2×10^3 cells/well/50 µL of growth medium, and preincubated overnight. The cells were treated with the indicated concentrations of afatinib for 96 h. An MTT assay was performed, OD_{570} values were measured using a microplate reader (see **Table of Materials**) and expressed as a percentage of the value obtained for the control cells. Data are presented as mean ± SEM of values from 6–12 replicate wells.

Figure 3: Established cells exhibited resistance to irreversible EGFR TKI, afatinib. Cells were seeded into a 96-well microplate at 2×10^3 cells/well/50 µL of growth medium and preincubated overnight. The cells were treated with the indicated concentrations of afatinib for 96 h. An MTT assay was performed, OD_{570} values were measured using a microplate reader (see **Table of Materials**) and expressed as a percentage of the value obtained for the control cells. Data are presented as mean ± SEM of values from 6–12 replicate wells.

Figure 4: Afatinib-resistant cell lines showed slower proliferation than parental PC-9 cells. Cells were seeded into 96-well microplates at 5×10^2 cells/100 µL/well. MTT assay was performed, and OD_{570} values were measured on days 0, 1, 2, 3, 5, and 7 using a microplate reader (see **Table of Materials**) and expressed as a percentage of the value obtained for the control cells. Data are presented as mean ± SEM of values from 6–12 replicate wells.

Figure 5: Gene copy number of EGFR was elevated in afatinib-resistant cells. The elevation of the EGFR gene copy number was measured by quantitative PCR of genomic DNA isolated from PC-9, AFR1, AFR2, and AFR3 cells.

Figure 6: Basal level of EGFR protein was increased in afatinib-resistant cells. Western blot analysis of phospho-EGFR, EGFR, HER2, HER3, and MET expression in PC-9, AFR1, AFR2, and AFR3 cells. β-Actin was used as a loading control.

Figure 7: DNA sequence reads in EGFR exons 19 and 20. Genomic DNA of PC-9, AFR1, AFR2, and AFR3 was amplified with specific primers for EGFR exon 19 and 21, and purified for sequencing.

DISCUSSION:

Here, we described a method for establishing three independent afatinib-resistant cell lines and characterized these cells by comparison to parental PC-9 cells. By stepwise dose escalation exposure, the parental PC-9 cells acquired resistance to afatinib over a period of 10–12 months. Clinically, the resistance mechanisms to EGFR TKIs are heterogeneous, and therefore, after the initial treatment with afatinib, PC-9 cells were divided into three independent p100 dishes and exposed further to afatinib. Initially, cell growth was not significantly slowed, but as the drug concentration approached the IC_{50} value, cell proliferation was slowed. This is a critical step for obtaining cells with acquired resistance to inhibitors. The proliferating cells should be split and transferred to new p100 dishes at a ratio of 1:10 or 1:5. When PC-9 cells were cultured in a p100 dish, some adherence was observed, but most cells grew in suspension. As the concentration of afatinib was increased, the cells adhered to the bottom of the tissue culture treated dishes. If most cells are adherent, they can be detached with a cell-scraper. The final concentration of afatinib was 1 μM, which is about 5 times the maximum serum concentration (C_{max})[14]. To obtain clear differences between parental and resistance clones, the final concentration was set to be higher than C_{max}.

One serious concern during the procedure is bacterial contamination, even though RPMI-1640 contains penicillin and streptomycin. To avoid this, two p100 dishes containing fresh growth medium can be prepared when the cells are split. When the cells reach the sub-confluent stage, the cells in one p100 dish can be further split, while the cells in the other p100 dish can be stored at -80 °C in cryopreservation medium (see **Table of Materials**) as a backup, such that if one line is contaminated, the stored line can be used.

It would be difficult to completely reproduce the acquisition of afatinib resistance in humans using cell culture. The emergence of the T790M mutation in EGFR exon 20 was reported as the dominant cause of resistance to afatinib. In our report, one resistant clone contained the T790M mutation[11]. Furthermore, the increase in wild-type EGFR, such as in AFR1 and AFR2 cells, has been reported by us and other groups[15,16]. The loss of the EGFR mutation and increase in wild type EGFR is also reported in clinical samples from patients with acquired resistance to EGFR-TKIs [17,18]. Therefore, in vitro studies of our current model may reflect the molecular profiles of clinical specimens with acquired resistance.

This stepwise dose escalation method is considered the most reliable for obtaining acquired resistant cells lines. However, initial high-dose afatinib exposure in cultured cells would likely better reflect the effects of afatinib treatment in patients with cancer, although establishing resistant cells is more difficult. Not only floating cell lines, such as PC-9 but also adherent cell lines, such as HCC827, 11-18, or HCC4006, can be employed for this method. This stepwise dose escalation method is also useful for establishing clones resistant to other inhibitors, using other cell lines, representing other types of cancer.

Exposure of parental cells to mutagenic agents, such as *N*-ethyl-*N*-nitrosourea, followed by selection of cells resistant to afatinib or osimertinib treatment has been reported to enable rapid acquisition of resistant clones [19,20]. However, this artificial method tends to cause specific base substitutions, such as GC to AT transitions and AT to TA transversions. Moreover, EGFR TKI is not a mutagenic agent in patients with NSCLC. Therefore, the method of stepwise dose escalation is more representative than using mutagenic agents.

Although EGFR TKIs are initially effective, cells eventually develop resistance to such single-target drugs, making it difficult to cure cancer. Inhibitors that target multiple molecules are therefore essential to develop. To this end, it is necessary to obtain cells with acquired resistance to multi-target inhibitors and evaluate the mechanisms underlying drug resistance.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

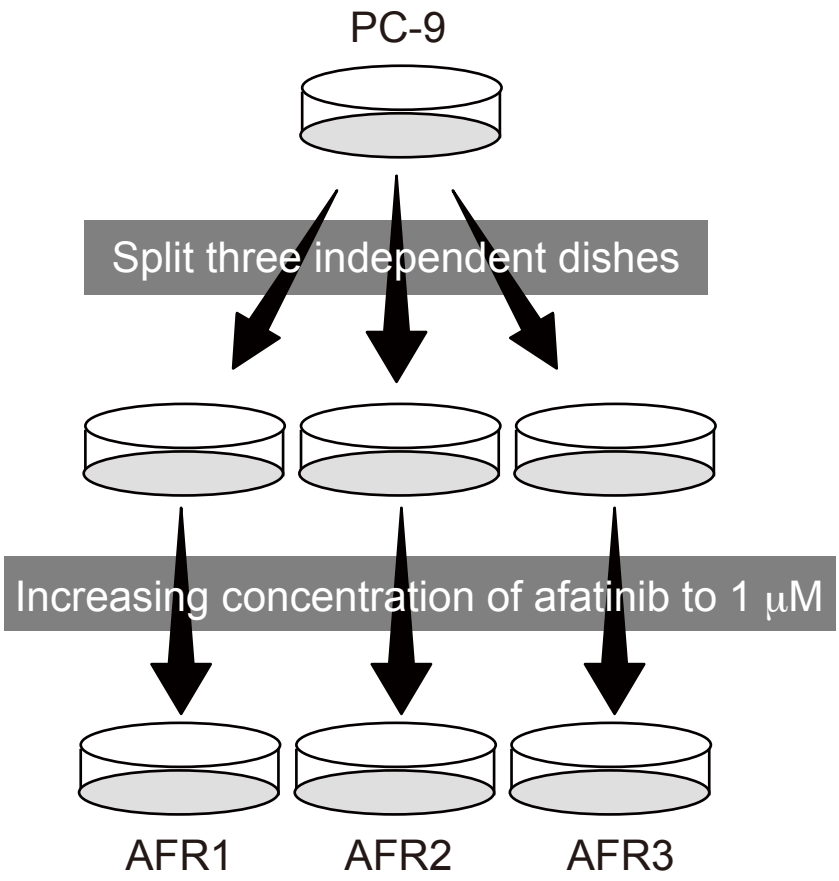


Figure 2

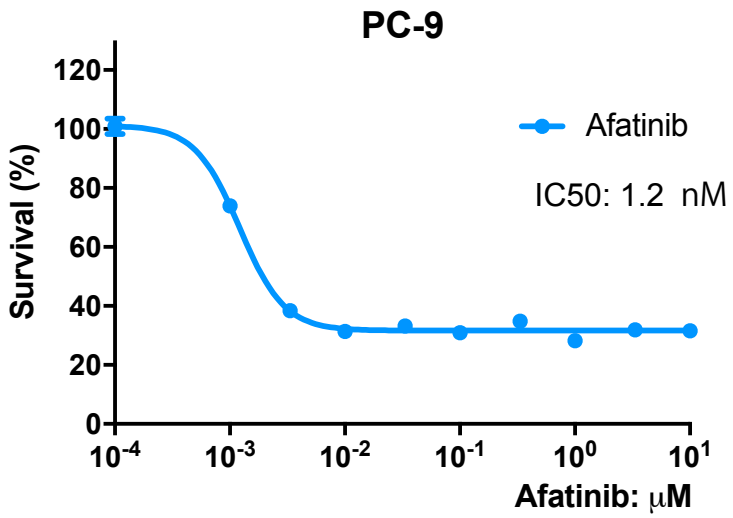
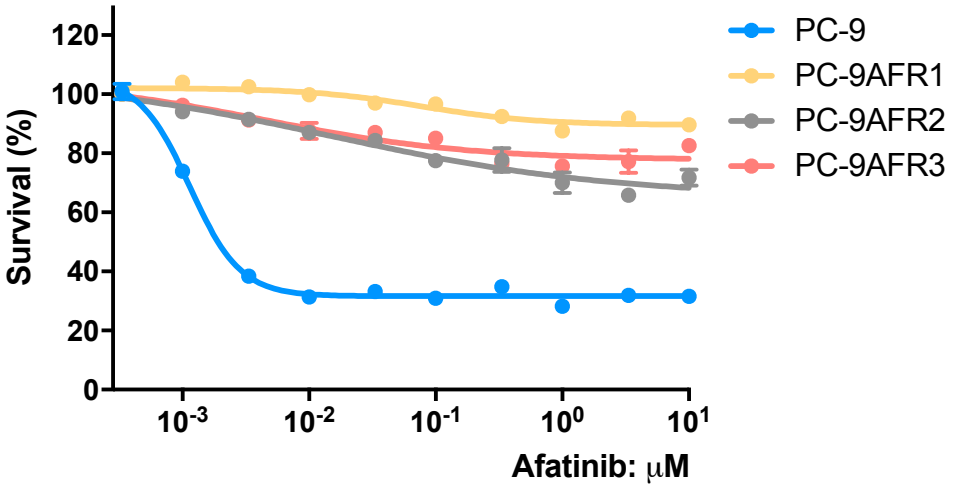


Figure 3



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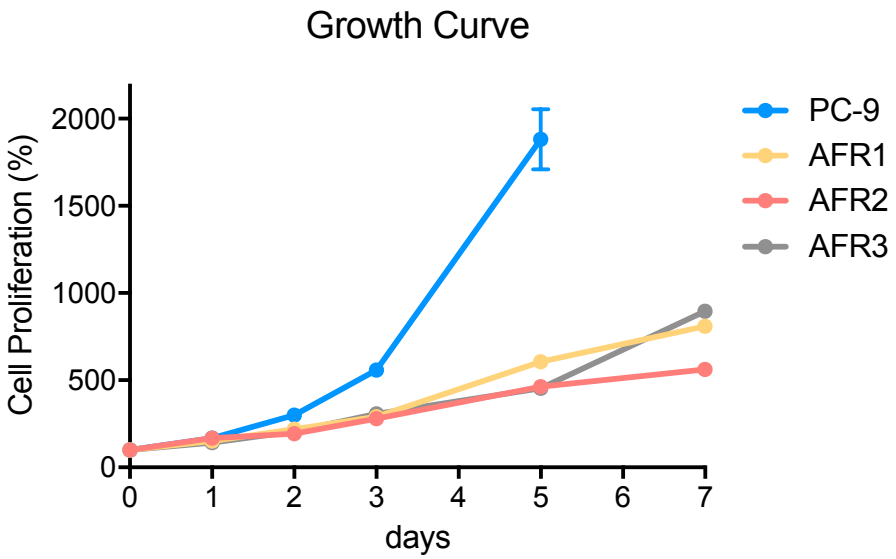

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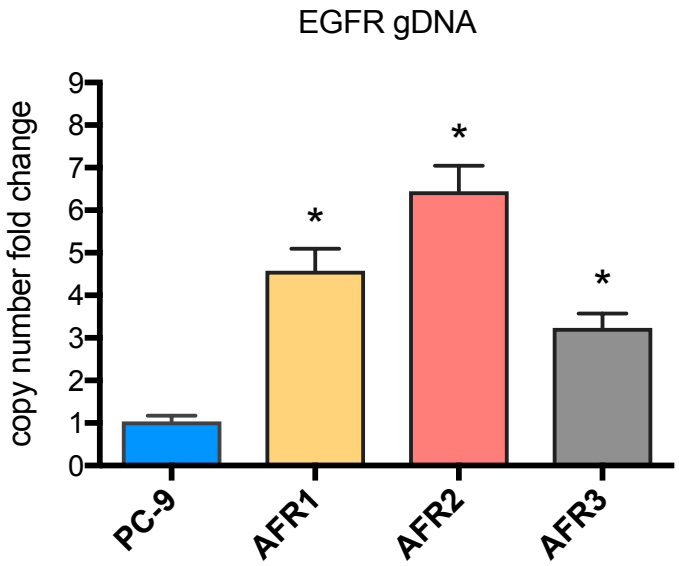


Figure 6

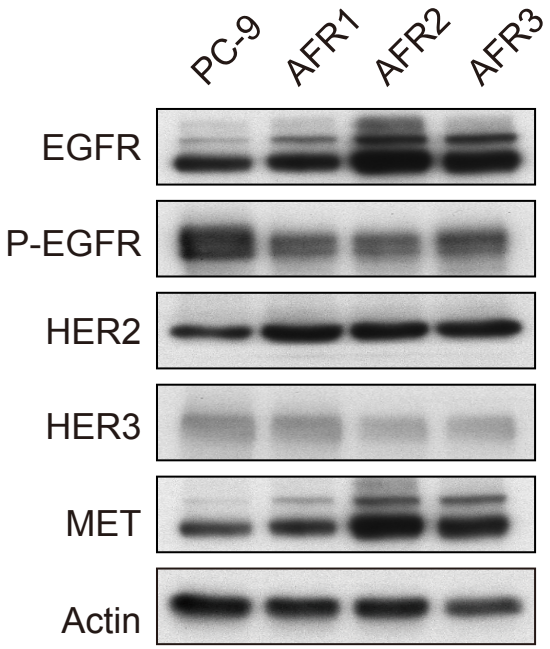
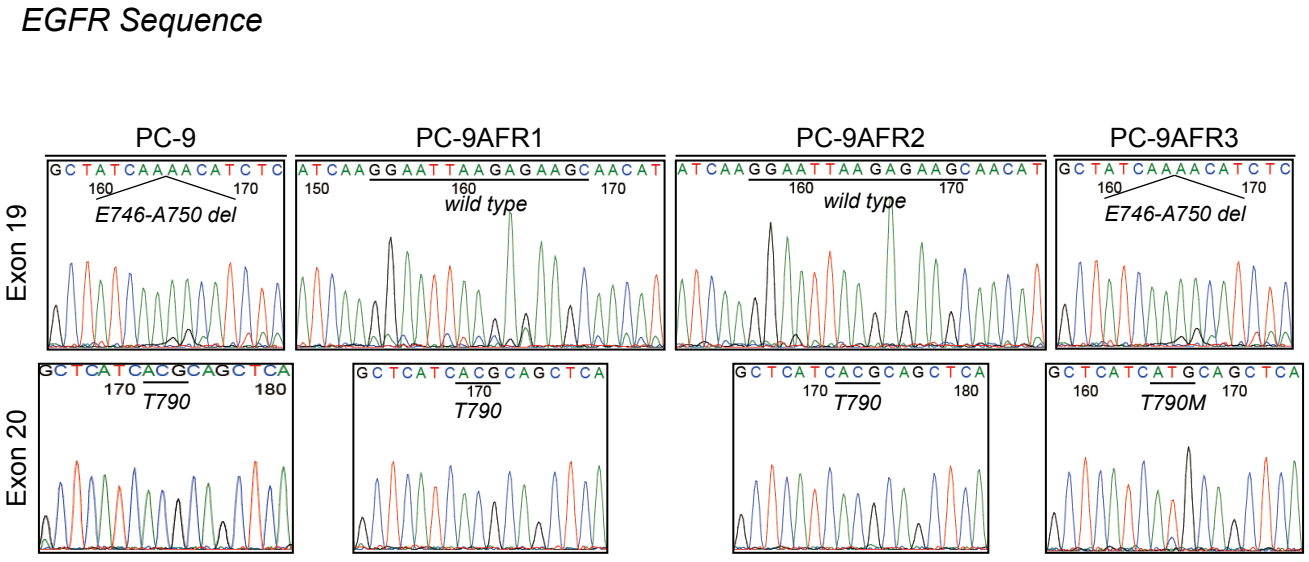


Figure 7



Name of Material/ Equipment	Company		Catalog Number	Comments/Description
afatinib	Selleck		S1011	
anti-EGFR monoclonal antibody	cell signaling technology		4267S	
bicinchoninc acid assay	sigma		B9643	
cell-culture treated 10cm dish	Violamo		2-8590-03	
CELL BANKER1	TakaRa		CB011	cryopreservation media
CellTiter 96	Promega		G4100	Non-Radioactive Cell Proliferation Assay; Dye solution and Solubilization/Stop solution
DMSO	Wako		043-07216	
ECL solution	Perkin Elmer		NEL105001EA	
FBS	gibco		26140-079	
GeneAmp 5700	Applied Biosystems			fluorescence-based RT-PCR-detection system
GraphPad Prism v.7 software	GraphPad, Inc.			a statistical software
NanoDrop Lite spectrophotometer	Thermo			spectrophotometer
Nonfat dry milk	cell signaling technology	9999S		
Pen Strep	gibco		15140-163	
phosphatase inhibitor cocktail 2	sigma		P5726	
phosphatase inhibitor cocktail 3	sigma		P0044	
Powerscan HT microplate reader	BioTek			
Power SYBR Green master mix	Applied Biosystems			SYBR Green master mix
protease inhibitor cocktail	sigma		P8340	
QIAamp DNA Mini kit	Qiagen		51306	DNA purification kit
QIAquick PCR Purification Kit	QIAGEN			PCR purification kit
RPMI-1640	Wako		189-02025	with L-Glutamine and Phenol Red
TBST powder	sigma		T9039	
Trans-Blot SD Semi-Dry Electrophoretic Transfer cell	Bio-Rad			semi-dry t4ransfer apparatus
96 well microplate	Thermo		130188	

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Title of Article: Establishment and characterization of three afatinib-resistant NSCLC PC-9 cells treated with increasing doses of afatinib

Author(s): Toshimitsu Yamaoka, Motoi Ohba, Yuki Matsunaga, Junji Tsurutani, Tohru Ohmori

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CORRESPONDING AUTHOR

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Department:	Showa University
Institution:	Advanced Cancer Translational Research Institute
Title:	Associate Professor
Signature:	Toshimitsu Yamaoka
Date:	March 1, 2019

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March 17, 2019

Bing Wu, Ph.D.
Editorial Board Members & Editor
Journal of Visualized Experiments

Dear Dr. Wu and Members of the *JoVE* Editorial Board:

Thank you for reviewing our manuscript MS# JoVE59473, titled "
Establishment and characterization of three afatinib-resistant lung adenocarcinoma PC-9 cell lines developed with increasing doses of afatinib". We are delighted to know that the Editor is quite enthusiastic about this work. Our responses to the Scientific Review Editor are addressed in detail in the attached document. All changes to the revised manuscript are highlighted in yellow.

We much appreciate your consideration of our revised report for publication in the *Journal of Visualized Experiments*. We believe that addressing the Scientific Review Editor's comments has substantially improved the quality and impact of the manuscript. Please do not hesitate to contact us if you have further questions or points of clarification.

We look forward to hearing from you.

Sincerely,

Toshimitsu Yamaoka, MD, PhD
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Showa University
1-5-8 Hatanodai, Shinagawa-ku
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Yamaoka et al. “Establishment and characterization of three afatinib-resistant lung adenocarcinoma PC-9 cells developed with increasing doses of afatinib” JoVE59473R1

Response to the JoVE Scientific Review Editor's comments:

We thank the JoVE Scientific Review Editor for his/her overall evaluation and are pleased that the reviewer recommended changes to our protocol.

Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Response: Thank you very much for your suggestion. We have used the English Editing service provided by Editage. They helped us proofread the manuscript thoroughly.

2. Please avoid long steps/notes (more than 4 lines).
   Response: We agree with the Review Editor on avoiding long sentences and steps. We have revised and rearranged the protocols accordingly.

3. Step 1.1.1: Please write this step in the imperative tense.
   Response: We have changed this sentence to imperative.

4. 1.1.2: Please write this step in the imperative tense.
   Response: Thank you very much for your suggestion. We have changed this sentence to imperative.

5. 1.2.2: Please write this step in the imperative tense.
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6. 1.2.5: Please write this step in the imperative tense.
   Response: We have changed this sentence to imperative.

7. 2.4.1: Please write this step in the imperative tense.
   Response: We have changed this sentence to imperative.

8. Figure 7: Please add a short description of the figure in Figure Legend.
   Response: We appreciate your suggestions. The legend to Figure 7 was added.

9. Please do not abbreviate journal titles.
   Response: Thank you very much for your suggestion. We have made the required change.

Thank you again for considering the publication of our revised report in the *Journal of Visualized Experiments*. We believe that by addressing the Scientific Review Editor’s comments we have substantially improved the quality and impact of the manuscript. Please do not hesitate

to contact me if you have further questions or points of clarification