

# Construction of a novel cell-based assay for the evaluation of anti-EGFR drug efficacy against EGFR mutation

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**Abstract.** Epidermal growth factor receptor (EGFR) overexpression and EGFR-mediated signaling pathway dysregulation have been observed in tumors from patients with various cancers, especially non-small cell lung cancer. Thus, several anti-EGFR drugs have been developed for cancer therapy. For patients with known EGFR activating mutations (*EGFR* exon 19 in-frame deletions and exon 21 L858R substitution), treatment with an EGFR tyrosine kinase inhibitor (EGFR TKI; gefitinib, erlotinib or afatinib) represents standard first-line therapy. However, the clinical efficacy of these TKIs is ultimately limited by the development of acquired drug resistance such as by mutation of the gatekeeper T790 residue (T790M). To overcome this acquired drug resistance and develop novel anti-EGFR drugs, a cell-based assay system for EGFR TKI resistance mutant-selective inhibitors is required. We constructed a novel cell-based assay for the evaluation of EGFR TKI efficacy against EGFR mutation. To this end, we established non-tumorigenic immortalized breast epithelial cells that proliferate dependent on EGF (MCF 10A cells), which stably overexpress mutant EGFR. We found that the cells expressing EGFR containing the T790M mutation showed higher resistance against gefitinib, erlotinib and afatinib compared with cells expressing wild-type EGFR. In contrast, L858R mutant-expressing cells exhibited higher TKI sensitivity. The effect of T790M-selective inhibitors (osimertinib and rociletinib) on T790M mutant-expressing cells was significantly higher than gefitinib and erlotinib. Finally, when compared with commercially available isogenic MCF 10A

cell lines carrying introduced mutations in EGFR, our EGFR mutant-overexpressing cells exhibited obviously higher responsiveness to EGFR TKIs depending on the underlying mutations because of the higher levels of EGFR phosphorylation in the EGFR mutant-overexpressing cells than in the isogenic cell lines. In conclusion, we successfully developed a novel cell-based assay for evaluating the efficacy of anti-EGFR drugs against EGFR mutation.

## Introduction

Epidermal growth factor receptor (EGFR) is a tyrosine kinase (TK) receptor that is activated by binding with ligands such as EGF and other growth factors. Activation of EGFR leads to the triggering of several downstream pathways including RAS/MAPK, PI3K/Akt and JAK/STAT pathways that regulate cell proliferation, survival, adhesion, migration, and differentiation (1). Overexpression of EGFR and dysregulation of EGFR-mediated signaling pathways have been observed in tumors from patients with various cancers, especially with non-small cell lung cancer (NSCLC), contributing to tumorigenesis and leading to a poor prognosis (2). Thus, EGFR is a promising molecular target as a therapeutic agent for patients with NSCLC and several anti-EGFR drugs have been developed for this purpose.

Lung cancer is the second most common cancer and remains the leading cause of cancer-related mortality worldwide. Notably, the majority of lung cancers (over 85% of patients) are categorized as NSCLC (3). Traditionally, lung cancer has been treated with surgery, radiation, and chemotherapy (4). Although chemotherapy has been the standard of care for patients with NSCLC, current clinical efforts are directed at molecular target drugs to improve outcome and reduce toxicity. Gefitinib, which primarily functions as an EGFR tyrosine kinase inhibitor (TKI), was approved in Japan for the treatment of NSCLC in 2002 and in the USA in 2003. In 2004, the presence of somatic mutations in the TK domain of EGFR was identified in patients with NSCLC responding to gefitinib (5,6). These mutations, consisting of in-frame deletions in *EGFR* exon 19 and the L858R substitution in exon 21,

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were associated with both *in vitro* sensitivity to gefitinib and therapeutic efficacy and are commonly referred to as 'activating mutations' as the mutant products are constitutively activated and oncogenic (1,7). Together, these mutations constitute 80-90% of all EGFR mutations in NSCLC. In addition, mutations involving G719 and L861 are also associated with gefitinib sensitivity, but their incidence is much lower (7). Thus, for patients with known EGFR activating mutations, treatment with an EGFR TKI (gefitinib, erlotinib or afatinib) represents current standard first-line therapy (8,9).

However, the clinical efficacy of gefitinib and erlotinib is ultimately limited by the development of acquired drug resistance such as by mutation of the gatekeeper T790 residue (T790M), which is the most frequent of acquired resistance mutations occurring in ~60% of patients after treatment with EGFR TKIs (1,9). Therefore, several EGFR TKIs have been developed for overcoming this acquired resistance to gefitinib and erlotinib. The so-called 'second-generation' of EGFR TKIs, which irreversibly and covalently bind with the catalytic site of the EGFR TK domain and widely inhibit TK receptors of the ErbB family (of which EGFR is a member), have been examined in clinical trials (1,9). However, despite promising preclinical evidence of activity against EGFR-mutated cell lines harboring the T790M mutation (10-12), the second-generation inhibitors (afatinib, neratinib and dacomitinib) did not demonstrate significant activity in patients harboring the T790M mutation (13-15). Consequently, to overcome the limitations of the second-generation inhibitors, a novel class of mutant-selective 'third-generation' inhibitors has been developed. Among these, rociletinib (16,17) and osimertinib (AZD9291) (18,19), which irreversibly and covalently inhibit the T790M resistance mutation as well as the activating mutations (exon 19 deletions and L858R), showed activities against T790M-positive NSCLC in clinical trials.

An efficient cell-based assay system for the identification of clinically efficacious EGFR mutant-selective inhibitors is required. Although the cell-based assays with human EGFR-mutated cell lines have been already reported (20-22), the activity against currently utilized EGFR-mutated cell lines harboring the T790M mutation is inconsistent with activity of the agents in patients harboring the T790M mutation. In addition, although the assay systems with EGFR mutant-overexpressing murine cell lines for EGFR TKIs have been reported (23-25), the assay systems with a human cell line have been not reported yet. Thus, we have developed a novel cell-based assay with a human non-tumorigenic epithelial cell line for the evaluation of anti-EGFR drug efficacy against EGFR mutation. Wild-type, T790M mutant, and L858R mutant *EGFR* genes were introduced into human non-tumorigenic immortalized breast epithelial MCF 10A cells that exhibit EGF-dependent growth using a retrovirus system to effect overexpression. To predict the construct validity of our system, the activity of EGFR TKIs including first, second and third-generation agents was evaluated utilizing these EGFR mutant-expressing cells in comparison to currently utilized isogenic lines.

## Materials and methods

**Compounds.** The 21 EGFR TKIs of the first, second and third-generation were used in this study (Table I). The stock

solutions (10 mM) of the compounds were prepared in dimethyl sulfoxide (DMSO) and stored at -80°C until use. The stock solutions were arrayed in 384-well plates and serially diluted 3 times to yield a concentration range from 10 mM to 52 nM. The purity and integrity of all compound solutions were measured using ultra performance liquid chromatography-mass spectrometry (Waters, Milford, MA, USA) as follows: a Waters CORTECS C18 column (1.6  $\mu$ m, i.d. 2.1x50 mm) was developed with an aqueous acetonitrile containing a 0.1% formic acid linear gradient system (5-90% MeCN, 1.6 min; flow rate, 1 ml/min), verifying the UV adsorption and mass of the major UV peaks (Table I).

**Construction of retroviral plasmids containing the mutant genes.** A wild-type *EGFR* cDNA clone was obtained from our human proteome expression resource library (HuPEX) (26). *EGFR* mutations were introduced into the wild-type cDNA using the QuickChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). pRetro-GW-IH, which was produced from pRetroX (Takara Bio, Shiga, Japan), was used as the retroviral vector. Wild-type and mutant EGFR constructs were transferred to the pRetro-GW-IH retroviral expression vector using the Gateway LR reaction (Thermo Fisher Scientific, Waltham, MA, USA).

**Cell culture.** MCF 10A cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The isogenic MCF 10A cell lines with EGFR mutations (L858R/+ and T790M/+) and their parental cells, used to create the mutant isogenic cells, were obtained from Horizon Discovery (Cambridge, UK). The GP2-293 packaging cell line was provided by Takara Bio.

MCF 10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Wako, Tokyo, Japan) supplemented with 5% heat-inactivated horse serum (Gibco, Thermo Fisher Scientific), 10  $\mu$ g/ml insulin (human, recombinant), 5  $\mu$ M forskolin (both from Wako), 0.5  $\mu$ g/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 20 ng/ml EGF (human, recombinant), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (all from Wako) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. GP2-293 packaging cells were cultured in DMEM (Wako) containing 10% heat-inactivated fetal bovine serum (Gibco) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Cell number and viability were measured using trypan blue dye exclusion with a Vi-Cell counter (Beckman-Coulter, Brea, CA, USA). To estimate confluency and doubling time (DT), the cells were seeded in 96-well plates (cat. CLS3595; Corning Inc., Corning, NY, USA) at 5x10<sup>3</sup> cells/well and measured using an InCuCyte ZOOM live cell imaging system (Essen BioScience, Ann Arbor, MI, USA) and InCuCyte ZOOM software.

**Retroviral packaging and infection.** GP2-293 packaging cells were seeded into 6-well plates at a density of 4x10<sup>5</sup> cells/well. The next day, the cells were transfected with 0.2  $\mu$ g plasmid containing the ecotropic envelope (*env*) gene and 2  $\mu$ g retroviral plasmid mixed with 7.2  $\mu$ g polyethylenimine (cat. 24765-2; Polysciences, Warrington, PA, USA). The culture supernatant was replaced with fresh medium at 7.5 h after transfection and again the following day. At 60 h after transfection, the

Table I. EGFR TKIs used in this study.

Compound	Generation	Supplier	Purity (%)
Erlotinib	First	Carbosynth	100
Gefitinib	First	Chemscene	100
AEE-788	First	Active Biochem	100
AG-1478	First	Selleck	100
Icotinib	First	Selleck	100
WHI-P154	First	Selleck	100
PD 153035	First	Selleck	100
Afatinib	Second	Selleck	100
Lapatinib	Second	LC Laboratories	100
AC 480	Second	Selleck	100
Dacomitinib	Second	Selleck	100
Pelitinib	Second	Selleck	100
Varlitinib	Second	Selleck	97.7
AZD 8931	Second	Selleck	100
AST-1306	Second	Selleck	95.2
WZ3146	Third	Selleck	100
WZ4002	Third	Selleck	100
WZ8040	Third	Selleck	100
Osimertinib (AZD9291)	Third	MedChem	100
Rociletinib	Third	MedChem	100
CUDC-101	Multitarget <sup>a</sup>	Selleck	100

<sup>a</sup>CUDC-101 also shows inhibition against histone deacetylase, EGFR, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitor.

virus-containing culture supernatant was harvested and centrifuged at 1,000 x g for 5 min to remove cell debris. This was used as the virus solution.

MCF 10A cells (ATCC) expressing the ecotropic receptor gene were seeded into 6-well culture plates at a concentration of  $7 \times 10^5$  cells and were infected with 1 ml appropriately diluted virus solution containing 8  $\mu\text{g/ml}$  hexadimethrine bromide (Sigma) the following day. At 24 h after infection, the cells were expanded onto a 10-cm dish and cultured in medium containing 20  $\mu\text{g/ml}$  hygromycin B (Wako).

**Measurement of total and phosphorylated EGFR.** Total and phosphorylated EGFR levels were detected by immunofluorescence staining. Cells were seeded at  $4 \times 10^3$  cells/well onto 384-well bottom clear black plates (cat. 781096; Greiner Bio-One, Frickenhausen, Germany). After 24 h culture, the cells were fixed with 4% paraformaldehyde phosphate buffer solution (Wako). After blocking with 5% bovine serum albumin in Tris-buffered saline solution for 30 min, the cells were incubated with primary antibodies against EGFR (mouse monoclonal clone AT2H8) and phospho-(Y1173)-EGFR (rabbit monoclonal clone E124) (both from Abcam, Cambridge, MA, USA) in 1:100 dilution at 37°C for 4 h. The cells were then washed 3 times with Tris-buffered saline containing 0.05% Tween-20 solution followed by incubation with anti-mouse or anti-rabbit IgG antibodies conjugated to DyLight488 or DyLight594 (NovusBio, Littleton, CO, USA), respectively,

in 1:1,000 dilution and stained with Hoechst 33342 solution (Dojindo Co., Kumamoto, Japan) in 1:1,000 dilution to visualize the nuclei at 37°C for 1 h. The cells were imaged with a x10 objective and a fluorescein filter using the Operetta High Content Imaging system and analyzed with the Columbus Image Data Storage and Analysis system (both from PerkinElmer, Waltham, MA, USA) to identify the nucleus, EGFR and phospho-EGFR.

To test the inhibition of EGFR phosphorylation mediated by EGFR TKIs, the cells were seeded at  $2 \times 10^4$  cells/well onto 96-well bottom clear black plates (cat. 6005182; PerkinElmer), cultured for 24 h, and then treated with gefitinib, afatinib, or osimertinib for 1 h. The cells were then fixed and stained as indicated above. The vehicle solvent (DMSO) was used as a control at a concentration of 0.1%.

**Growth inhibition assay.** The growth inhibitory activity of EGFR TKIs against cells was assayed by measuring the amount of ATP in the cells using CellTiter-Glo (Promega, Madison, WI, USA). The cells were incubated in 384-well plates at a density of  $1 \times 10^3$  cells/well with a medium volume of 40  $\mu\text{l}$  for 4 h. The cells were then treated with 0.1  $\mu\text{l}$  EGFR TKI solutions at final concentration ranges of 25  $\mu\text{M}$  to 1.3 nM (10-point dose) using an ADS-348-8 Multistage-dispense station (Biotec, Tokyo, Japan). The vehicle solvent (DMSO) was used as a control at a maximum concentration of 0.25%. After 72 h, 10  $\mu\text{l}$  CellTiter-Glo reagent solution was added to the medium and the plate was mixed with a plate mixture and incubated for 10 min at 30°C. The luminescence was measured using an EnSpire plate reader (PerkinElmer). The IC<sub>50</sub> values were analyzed using Morphit software (The Edge Software Consultancy, Guildford, UK).

## Results

**Cells overexpressing EGFR mutant genes.** To develop a cell-based assay system for evaluating EGFR TKIs, MCF 10A cells (ATCC), which are non-tumorigenic immortalized breast epithelial cells and EGF proliferation-dependent, were used in this study. Firstly, MCF 10A cells expressing ecotropic receptor were created and used as a control to establish MCF 10A cells overexpressing wild-type EGFR (WT) and EGFR mutants (L858R and T790M) using a retroviral system. The levels of total and phosphorylated EGFR (pY1173) were detected with immunofluorescence. As predicted, the mean intensities of green fluorescence corresponding to EGFR in the overexpressing cells were ~1.5 times higher than that of the MCF 10A cell line expressing the ecotropic receptor (Fig. 1A). The scattering in the EGFR expression histograms was narrow (SD, 195-451), suggesting that the amount of EGFR in the cells was constant. The levels of phosphorylated EGFR (red) in WT and T790M cells were ~2 times that of the control line, whereas the constitutively active EGFR mutant L858R showed a 3-fold increase compared with the control line (Fig. 1A). However, the scattering in the histograms of the phosphorylated EGFR levels in the gene-overexpressing cells was wide (SD, 1017-1707), suggesting that phosphorylated EGFR accumulated in the cells over time.

Next, we examined whether the depletion of EGF affected the proliferation of the various cells. The cells were cultured

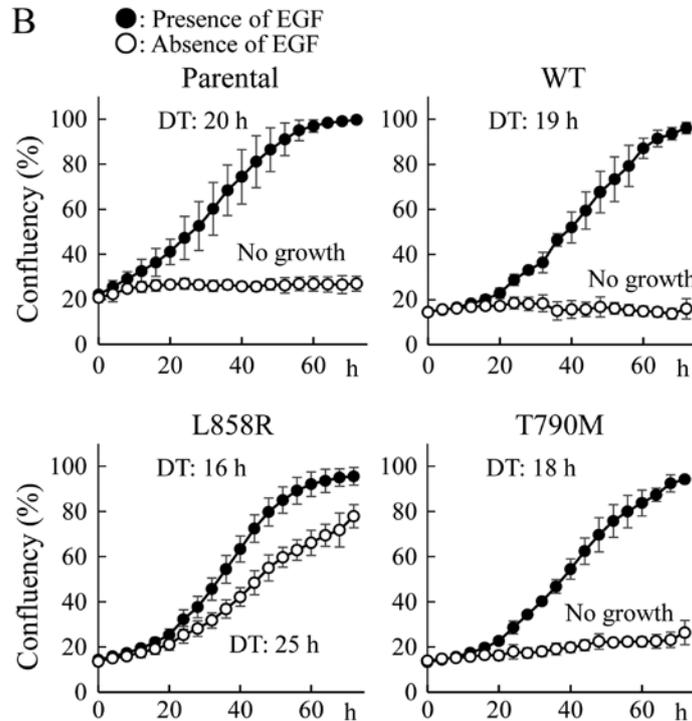
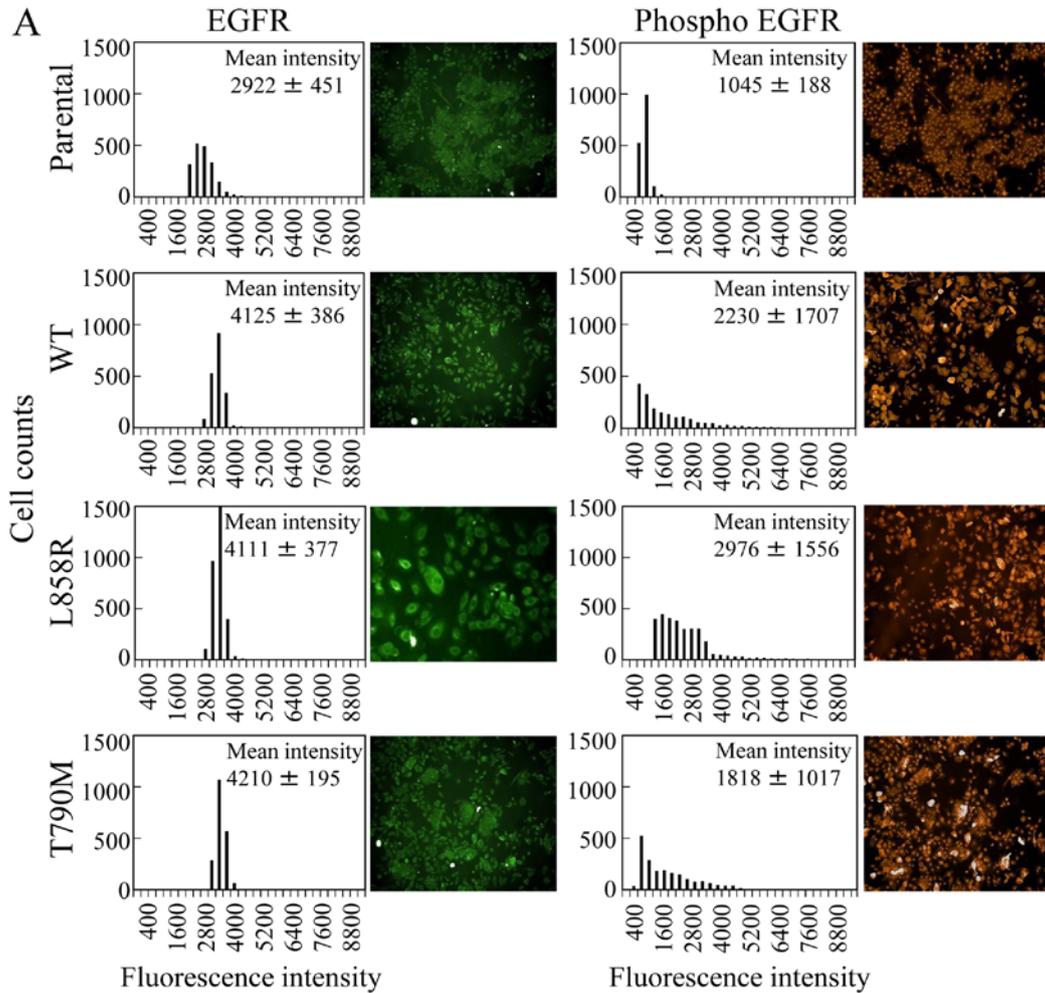


Figure 1. Evaluation of the control MCF 10A line and the cells overexpressing WT, L858R and T790M epidermal growth factor receptor (EGFR). (A) Levels of EGFR expression and its phosphorylation as measured using immunofluorescence. The fluorescence intensities of randomly selected 2,500 cells in 3 wells are shown as histograms in units of 400. The data presented are the means  $\pm$  SD (n=2,500). The fluorescence images using x10 objective were electronically overlaid. (B) EGF dependency of cell growth in EGFR mutant-expressing cells. Cell images were captured with InCuCyte ZOOM live cell imaging system every 4 h and the doubling time (DT) of the cells was calculated. Each data point represents the means  $\pm$  SD (n=9) of three independent experiments.

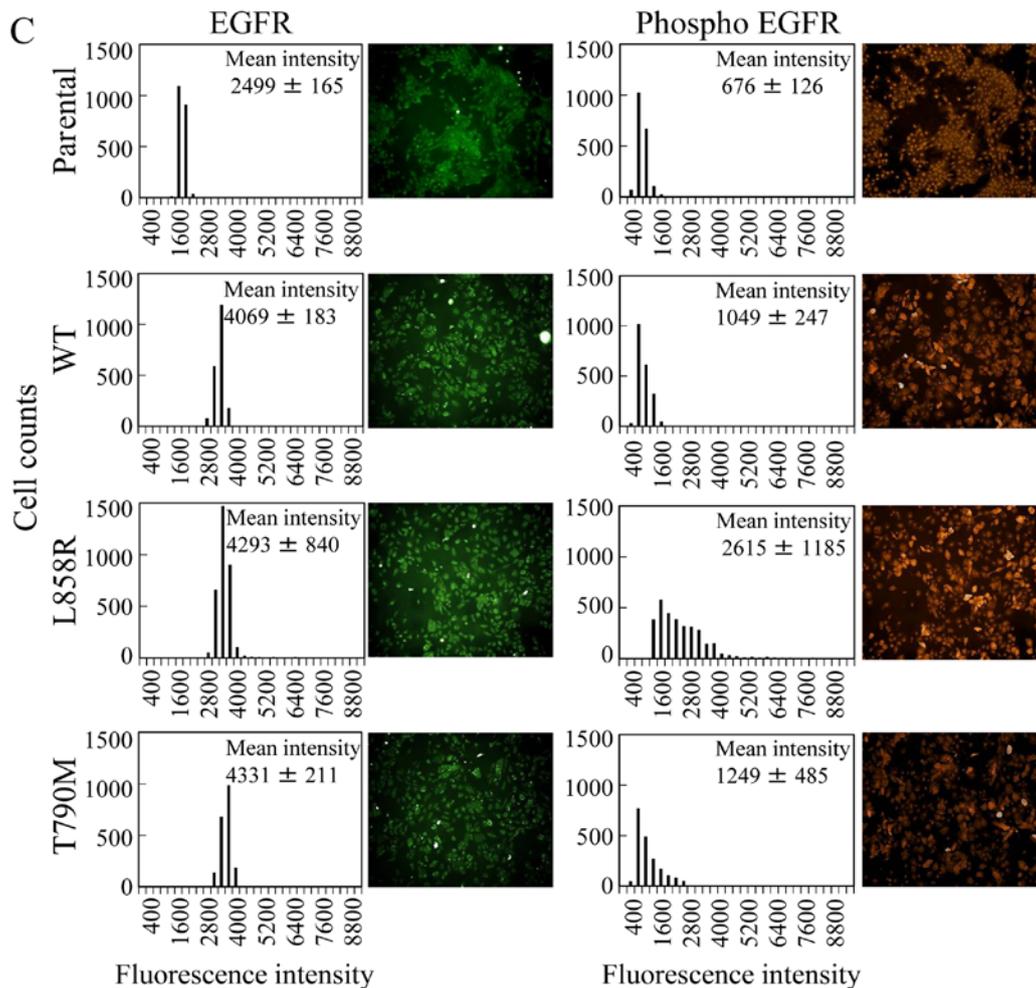


Figure 1. Continued. (C) Levels of EGFR expression and its phosphorylation in cells without EGF stimulation. The cells were cultured in 384-well plates. The medium was replaced with EGF-free medium at 24 h after seeding and the cells were cultured for 3 h.

in medium with or without EGF for 72 h. The confluency and DT of the cells were measured using the IncuCyte ZOOM live cell imaging system. DTs of WT, L858R, and T790M (16-19 h) cells were similar to the control line (20 h) in medium with EGF (Fig. 1B). In the absence of EGF, the control, WT and T790M lines did not grow, indicating that their growth is dependent on EGF (Fig. 1B). On the other hand, the constitutively active EGFR mutant L858R was able to proliferate in the absence of EGF; DTs of L858R with and without EGF were 16 and 25 h, respectively.

To confirm the relationship between EGFR phosphorylation and EGF stimulation, the cells were cultured without EGF for 3 h and the phosphorylated receptor levels were measured (Fig. 1C). A high level of phosphorylated EGFR was found in the L858R overexpressing line that exhibits growth under these conditions of EGF depletion as was observed in culture with EGF, whereas the levels of phosphorylated EGFR of the other cells were low compared with the respective EGF-containing cultures (Fig. 1C).

*Growth inhibitory effects of EGFR TKIs depend on the underlying EGFR mutation.* To investigate the sensitivity of EGFR TKIs to L858R and T790M, a growth inhibitory assessment was conducted for the various cells treated with these

agents. Each generation of EGFR TKIs under clinical use and in development was examined in these experiments (Table I). The cells were treated with the drugs at 4 h after seeding and incubated for 72 h. Then, the numbers of viable cells were calculated by measuring the amounts of ATP in the cells using CellTiter-Glo reagents. The  $IC_{50}$  values of 21 EGFR TKIs against each cell type are shown in Table II. WT demonstrated greater resistance against all EGFR TKIs than the control cells. The  $IC_{50}$  values of the drugs against WT were 28 nM-4.5  $\mu$ M. Afatinib, which is a representative second-generation drug, exhibited the strongest inhibitory effect in 21 EGFR TKIs. No major difference was observed in the inhibitory effects between each generation of EGFR TKIs.

Next, the sensitivity of EGFR TKIs to L858R and T790M was evaluated. The relative ratios (mutant/WT) of the  $IC_{50}$  values of L858R and T790M were calculated (Table II). The  $IC_{50}$  values of all drugs against the constitutively active EGFR mutant L858R were significantly lower than those of the control and WT lines. In addition, these high sensitivities (ratios, 0.06-0.18) were especially evident in the first and third-generations of EGFR TKIs. On the other hand, T790M exhibited remarkable resistance against the first-generation agents. Although the relative ratios of the second-generation varied (1.68-104.58), a weaker inhibitory effect was observed

Table II. IC<sub>50</sub> values of EGFR TKIs against control- and various EGFR-expressing cell lines.

Compound	Generation	Control	WT	L858R	T790M
Erlotinib	First	0.221	0.379	0.031 (0.08)	4.315 (11.39)
Gefitinib	First	0.180	0.371	0.034 (0.09)	5.781 (15.58)
AEE-788	First	0.157	0.342	0.032 (0.09)	2.488 (7.27)
AG-1478	First	0.088	0.163	0.018 (0.11)	8.697 (53.36)
Icotinib	First	0.524	0.601	0.043 (0.07)	14.639 (24.36)
WHI-P154	First	0.267	0.447	0.080 (0.18)	8.110 (18.14)
PD 153035	First	0.095	0.190	0.021 (0.11)	8.382 (44.12)
Afatinib	Second	0.009	0.028	0.002 (0.07)	0.365 (13.04)
Lapatinib	Second	1.612	4.502	2.590 (0.58)	7.564 (1.68)
AC 480	Second	1.159	1.637	1.452 (0.89)	10.274 (6.28)
Dacomitinib	Second	0.011	0.037	0.002 (0.05)	1.060 (28.65)
Pelitinib	Second	0.014	0.059	0.005 (0.08)	0.189 (3.20)
Varlitinib	Second	1.550	2.483	1.217 (0.49)	8.331 (3.36)
AZD 8931	Second	0.013	0.040	0.004 (0.10)	4.183 (104.58)
AST-1306	Second	0.029	0.093	0.025 (0.27)	0.471 (5.06)
WZ3146	Third	0.114	0.309	0.028 (0.09)	0.156 (0.50)
WZ4002	Third	0.557	1.525	0.090 (0.06)	0.643 (0.42)
WZ8040	Third	0.096	0.285	0.016 (0.06)	0.102 (0.36)
Osimertinib	Third	0.197	0.628	0.065 (0.10)	0.419 (0.67)
Rociletinib	Third	0.754	1.667	0.246 (0.15)	0.778 (0.47)
CUDC-101	Multitarget	0.051	0.084	0.025 (0.30)	0.056 (0.67)

IC<sub>50</sub> values indicate  $\mu\text{M}$ . The inhibitory ratio of mutant/WT IC<sub>50</sub> values is shown in parentheses. EGFR, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitor.

Table III. IC<sub>50</sub> values of EGFR TKIs against the isogenic cells.

Compound	Generation	Parental	L858R	T790M
Erlotinib	First	0.282	0.051 (0.18)	1.750 (6.21)
Gefitinib	First	0.218	0.045 (0.21)	0.846 (3.88)
PD 153035	First	0.147	0.025 (0.17)	0.520 (3.54)
WHI-P154	First	0.362	0.112 (0.31)	1.427 (3.94)
Afatinib	Second	0.016	0.002 (0.13)	0.017 (1.06)
Lapatinib	Second	2.085	2.330 (1.12)	4.823 (2.31)
AC 480	Second	1.545	1.839 (1.19)	4.708 (3.05)
Dacomitinib	Second	0.023	0.003 (0.13)	0.031 (1.35)
Osimertinib	Third	0.323	0.084 (0.26)	0.301 (0.93)
Rociletinib	Third	1.167	0.303 (0.26)	0.504 (0.43)
CUDC-101	Multitarget	0.066	0.038 (0.58)	0.078 (1.18)

IC<sub>50</sub> values indicate  $\mu\text{M}$ . The inhibitory ratio of mutant/parental IC<sub>50</sub> values is shown in parentheses. EGFR, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitor.

against T790M than against the WT receptor. As expected, the inhibitory effect of the third-generation agents against T790M was the strongest compared to the first and second-generations.

*Inhibition of EGFR phosphorylation with EGFR TKIs depends on the underlying EGFR mutation.* Inhibition of

EGFR phosphorylation in WT, L858R and T790M by treatment with gefitinib, afatinib and osimertinib was analyzed by immunofluorescence. The cells were cultured on 96-well plates for 24 h, treated with EGFR TKIs for 1 h, fixed, and stained with the antibody against phosphorylated EGFR (pY1173). The inhibitory rates of EGFR phosphorylation in L858R markedly increased at low concentrations (3–30 nM) of gefitinib, afatinib and osimertinib compared with WT (Fig. 2). On the other hand, the levels of phosphorylated EGFR in T790M did not decrease at a high concentration (3  $\mu\text{M}$ ) of gefitinib or afatinib (Fig. 2), whereas a third-generation inhibitor, osimertinib, decreased the levels of phosphorylated EGFR in T790M to levels similar to WT (Fig. 2D). These results were identical to the growth inhibitory effects of these agents.

*Comparison between the overexpressing and isogenic cells with EGFR mutations.* To compare our mutant-expressing cells with the introduced mutant cells created by using isogenic cell line technology, we investigated the sensitivity of EGFR TKIs including each generation (Table III) toward the L858R and T790M mutant isogenic cells. First, we evaluated the levels of total and phosphorylated EGFR (pY1173) by immunofluorescence. The levels of total EGFR in the MCF 10A cell lines between two cell lines derived from ATCC and Horizon Discovery were different. The mean intensities of EGFR in the parental cells from Horizon Discovery (Fig. 3A) were  $\sim 1.5$  times higher than those of the control cells from ATCC (Fig. 1A). The mean intensities and histograms of

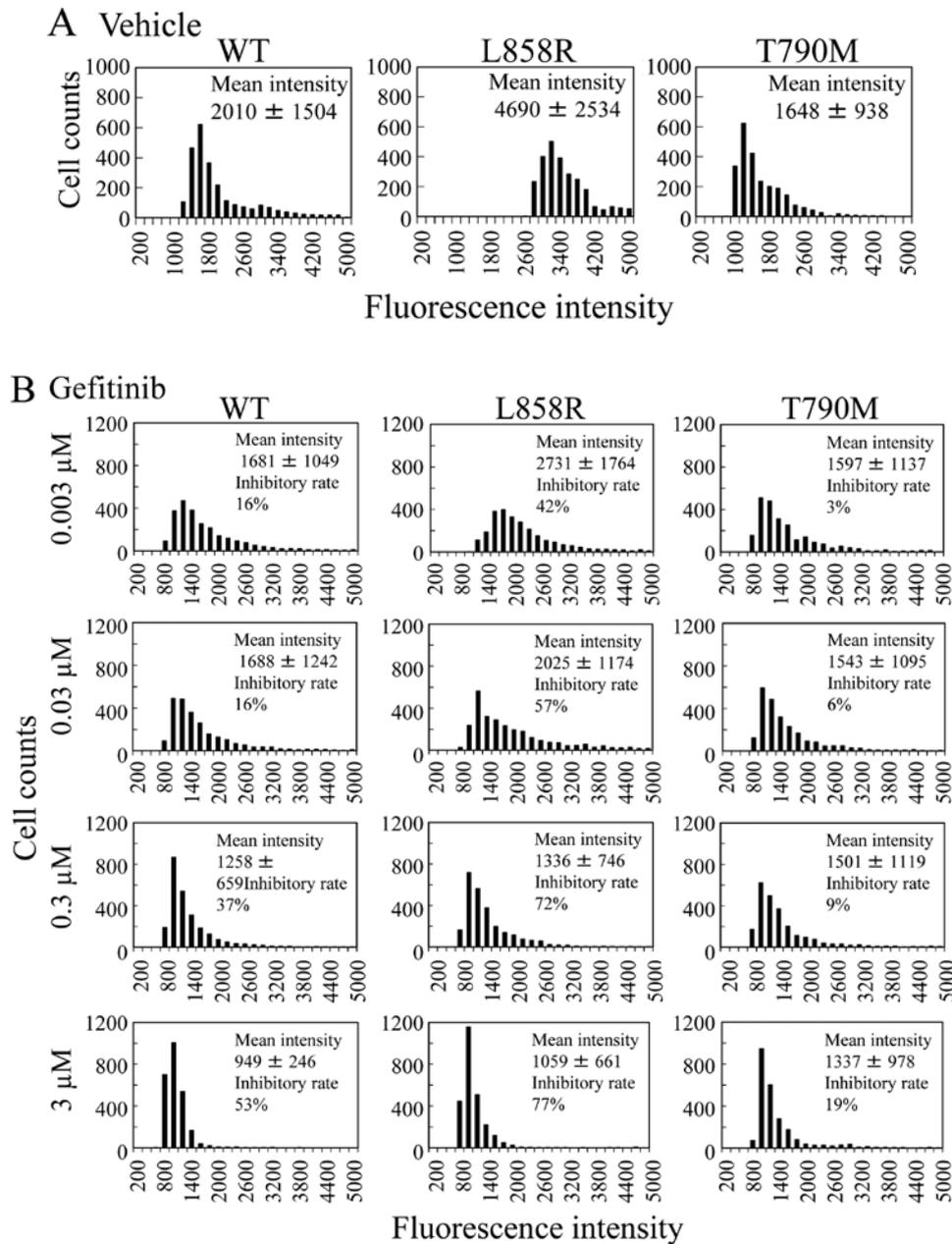


Figure 2. Inhibition of epidermal growth factor receptor (EGFR) phosphorylation by EGFR tyrosine kinase inhibitors (TKIs) in cells overexpressing WT, L858R and T790M EGFR. Levels of EGFR phosphorylation as measured using immunofluorescence are shown in cells treated with (A) vehicle solvent and (B) gefitinib. The fluorescence intensities of the randomly selected 2,500 cells are shown as histograms in units of 400. The data presented are the means  $\pm$  SD (n=2,500). The inhibitory rate (%) is [(vehicle - EGFR TKI)/vehicle] x100.

the fluorescence in the isogenic cells were similar to those of the parental cells (Fig. 3A). Although the levels of EGFR in the isogenic and mutant-expressing cells were equivalent, the levels of phosphorylated EGFR in the isogenic cells were lower than those in the mutant-expressing cells. Notably, the level of phosphorylated EGFR in the constitutively active EGFR mutant L858R did not increase, unlike the observation in the L858R-expressing cells.

Next, we examined whether the growth of isogenic cells was dependent on EGF. We found that the parental (MCF 10A from Horizon Discovery) and the T790M and L858R mutant isogenic lines showed EGF dependency. In contrast to the L858R-expressing cells, the L858R mutant isogenic cells did not grow without EGF unlike the L858R-expressing

cells (Fig. 3B), because the phosphorylated EGFR level in the L858R mutant isogenic cells was similar to that of the parental cells and was not elevated (Fig. 3A).

The growth inhibitory effects of 11 EGFR TKIs against parental and L858R and T790M mutant isogenic cells were tested. The IC<sub>50</sub> values are shown in Table III. Although the EGFR TKI sensitivities against the isogenic cells were similar to those of the mutant EGFR-expressing cells, the relative ratios (mutant/WT) of the IC<sub>50</sub> values differed. The IC<sub>50</sub> values of gefitinib against parental, L858R and T790M isogenic cells were 218, 45 and 846 nM, respectively (Table III and Fig. 3C). The relative ratios compared to parental cells were 0.21 for L858R and 3.88 for T790M (Table III). These results showed that the drug responsiveness against isogenic mutants was

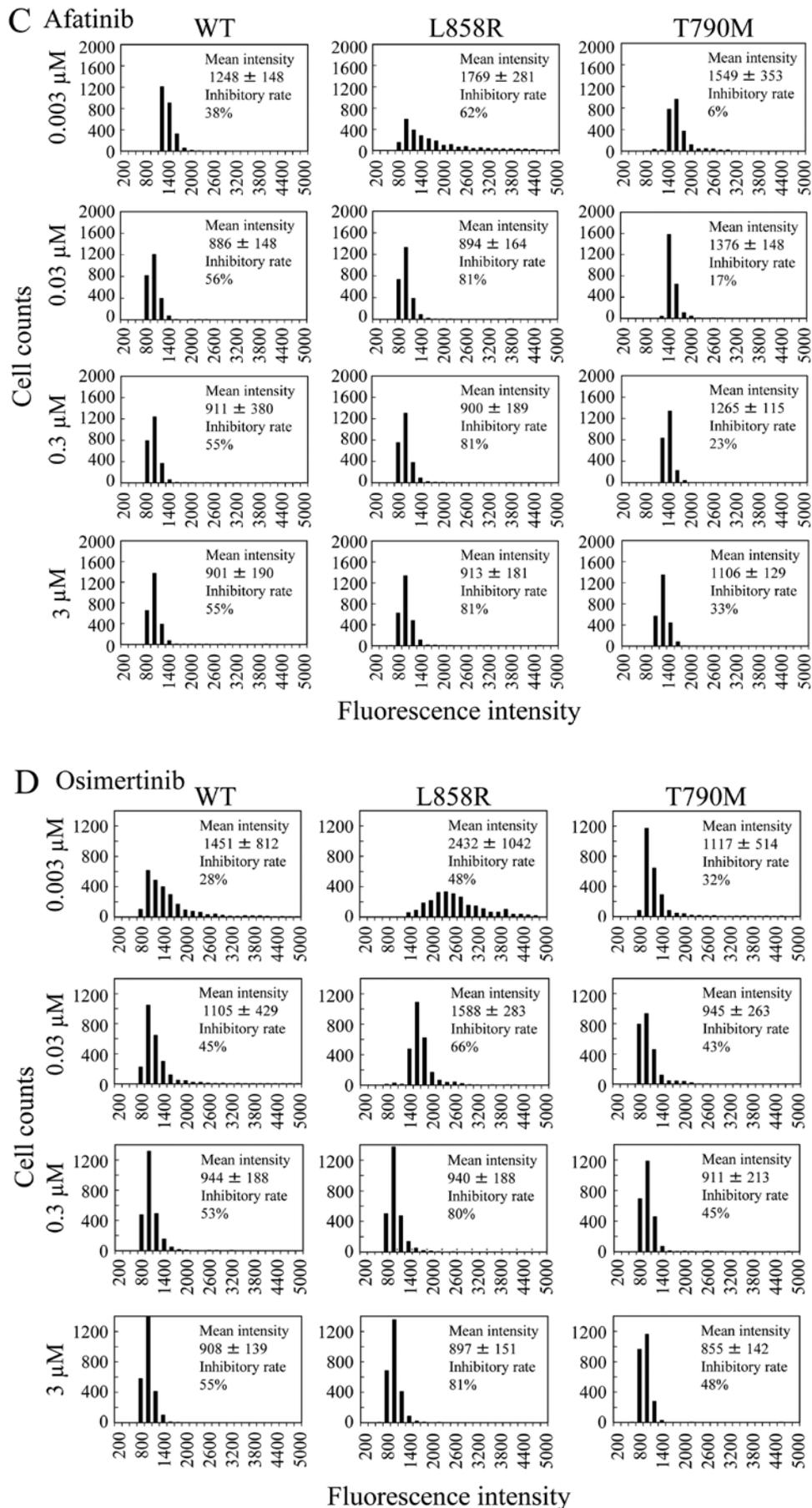


Figure 2. Continued. Levels of EGFR phosphorylation as measured using immunofluorescence are shown in cells treated with (C) afatinib and (D) osimertinib. The fluorescence intensities of the randomly selected 2,500 cells are shown as histograms in units of 400. The data presented are the means  $\pm$  SD ( $n=2,500$ ). The inhibitory rate (%) is  $[(\text{vehicle} - \text{EGFR TKI})/\text{vehicle}] \times 100$ .

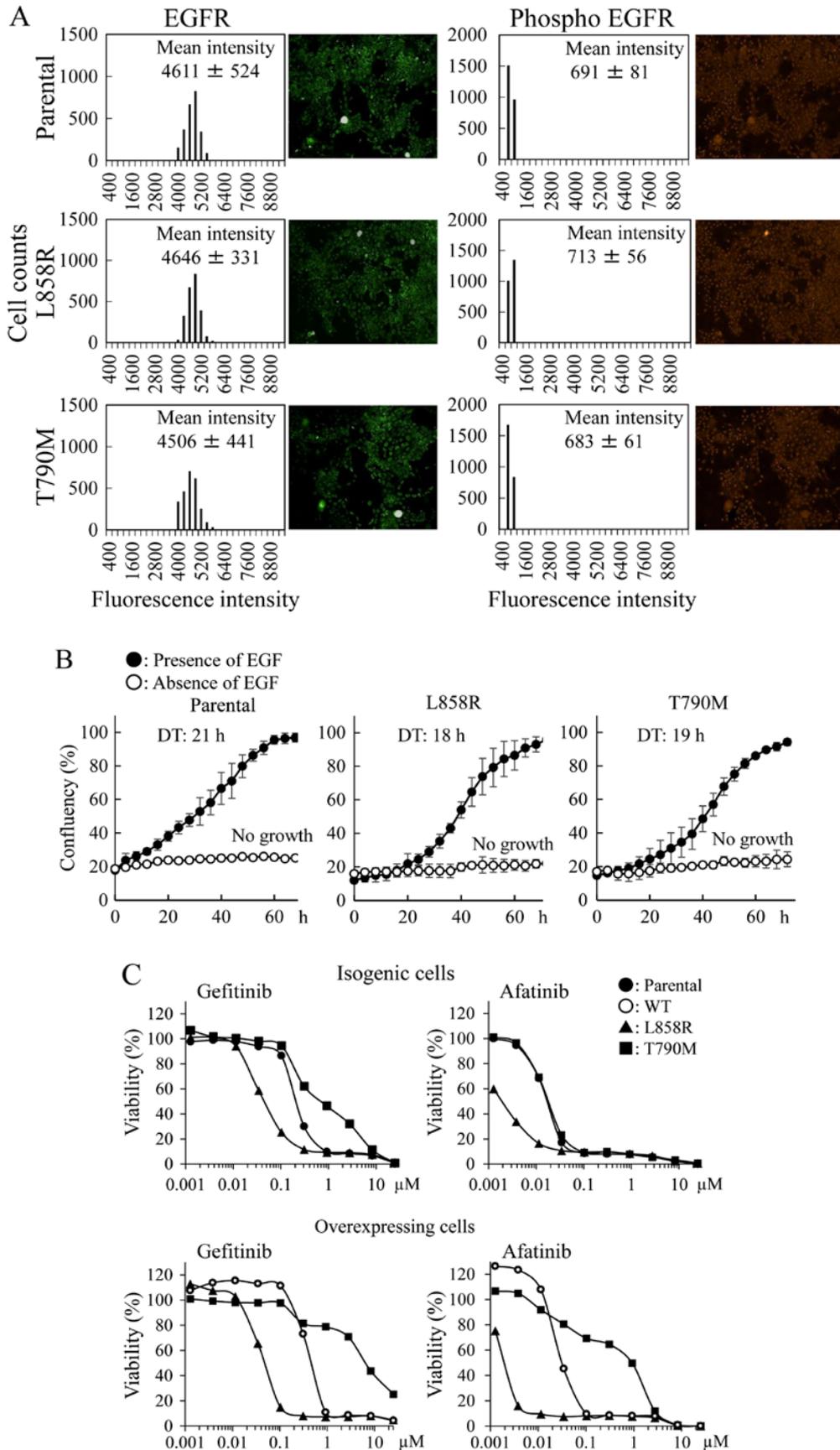


Figure 3. Comparison between the epidermal growth factor receptor (EGFR) mutation overexpressing and isogenic cells. (A) Levels of EGFR expression and its phosphorylation in isogenic cells. The fluorescence intensities of randomly selected 2,500 cells in 3 wells are shown as histograms in units of 400. The means ± SD (n=2,500) are presented. (B) Cell growth dependency on EGF of isogenic cells with mutations in EGFR. Confluency and doubling time were measured using the InCuCyte ZOOM live cell imaging system. Doubling time (DT) represents doubling time. Each data point represents the means ± SD (n=9) of three independent experiments. (C) Effect of EGFR tyrosine kinase inhibitors (TKIs) on the proliferation of MCF 10A cells overexpressing EGFR mutant genes and of isogenic MCF 10A cells with mutations in EGFR. The data are the means of duplicate experiments.

lower than that for mutant-expressing cells; 0.09 for L858R and 15.58 for T790M (Table II and Fig. 3C). In addition, the  $IC_{50}$  values of afatinib against the isogenic cells were 16 (parental), 2 (L858R), and 17 nM (T790M), demonstrating that the parental and T790M mutant isogenic cells exhibited similar values (Table III and Fig. 3C). On the contrary, the  $IC_{50}$  value (365 nM) of afatinib against T790M-expressing cells was higher than for cells expressing WT (28 nM) (Table II and Fig. 3C). These results indicated that our mutant gene-expressing cells may possess higher drug sensitivity than isogenic cells.

## Discussion

We established MCF 10A cells overexpressing EGFR mutants to construct a novel cell-based assay for the evaluation of EGFR TKIs, which could not be correctly evaluated (i.e., in a clinically relevant manner) using available T790M-mutated tumor cell lines or xenograft models based on these cell lines (10-15) because of differences in genetic backgrounds (e.g., genome sequence and gene expression) among WT and T790M-mutated tumor cell lines. Unlike in these cell lines, the comparative analysis between WT and mutant EGFR-expressing cells is straightforward. As expected, L858R-expressing cells showed an increase of EGFR TKI sensitivity compared with cells expressing WT, whereas T790M-expressing cells showed resistance against EGFR TKIs (Table II). In addition, the third generation of EGFR TKIs inhibited the cell growth of T790M-expressing cells that were more resistant to the first and second generation than were WT. For example, the third-generation agent osimertinib (currently marketed) exhibited substantially higher inhibitory activity against the cell growth of T790M-expressing cells than erlotinib and gefitinib. These results were identical with the clinical findings for these drugs (1,7), suggesting the possibility of evaluation reflecting the state of cancer tissue in an organism using this mutant-expressing cell line. Furthermore, our results suggest the additional possibility of the utility of this mutant EGFR-expressing line for the evaluation of mutant-selective inhibitors and drug screening.

The isogenic cell line technology used to develop the lines studied here was initially developed by Di Nicolantonio *et al*, wherein a panel of isogenic human cell lines was created by employing homologous recombination (27). The isogenic cells (T790M and L858R mutants) exhibited sensitivity toward EGFR TKIs as expected; however, our mutant gene-expressing cells exhibited higher drug sensitivity than these isogenic cells (Fig. 3C). In addition, L858R mutant isogenic cells did not show growth in medium without EGF (Fig. 3B). We consider that the difference in drug sensitivity and cell growth is due to the levels of mutant EGFR and of EGFR phosphorylation, as the EGFR mutant gene-expressing cells showed higher levels of EGFR expression and phosphorylation than the control line (Fig. 1), whereas mutant isogenic cell EGFR expression was equivalent to the parental line and phosphorylated EGFR did not accumulate (Fig. 3).

In conclusion, the results of this study demonstrate that our mutant gene-expressing cell model is superior to isogenic cells for the evaluation of anti-EGFR drug efficacy against EGFR mutation.

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