Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells

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Abstract:

Acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) is a critical problem in the treatment of lung cancer. While several mechanisms have been demonstrated to be responsible for acquired resistance, all mechanisms have not been uncovered. In this study, we investigated the molecular and cellular profiles of the acquired resistant cells to EGFR-TKI in EGFR mutant lung cancers. Four EGFR-mutant cell lines were exposed to gefitinib by stepwise escalation and high-concentration exposure methods, and resistant sublines to gefitinib were established. The molecular profiles and cellular phenotypes of these resistant sublines were characterized. Although previously reported alterations including secondary EGFR T790M mutation, MET amplification, and appearance of epithelial to mesenchymal transition (EMT) features were observed, these 2 drug-exposure methods revealed different resistance mechanisms. The resistant cells with EMT features exhibited down-regulation of microRNA-200c by DNA methylation. Furthermore, the HCC827-derived subline characterized by the high-concentration exposure method exhibited not only EMT features but also stem cell-like properties, including aldehyde dehydrogenase isoform 1 (ALDH1A1) overexpression, increase of side-population, and self-renewal capability. Resistant sublines with stem cell-like properties were resistance
to conventional chemotherapeutic agents but equally sensitive to histone deacetylase and proteasome inhibitors, compared with their parental cells. ALDH1A1 was up-regulated in clinical samples with acquired resistance to gefitinib. In conclusion, our study indicates that the manner of EGFR-TKI exposure influences the mechanism of acquired resistance and the appearance of stem cell-like property with EGFR-TKI treatment.
Introduction

Epidermal growth factor receptor (EGFR) mutations are oncogenic alterations in non-small cell lung cancer (NSCLC) (1, 2). First-generation EGFR-tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, have exhibited significant antiproliferative effects against NSCLC with EGFR mutations in preclinical studies (1, 2) and have also resulted in prolonged disease-free survival in randomized phase III studies (3-5). However, patients with EGFR-activating mutations who initially respond to EGFR-TKIs eventually acquire resistance, which is a critical problem in the treatment of patients with advanced NSCLC. Several mechanisms are believed to be responsible for acquired resistance to EGFR-TKI, including secondary EGFR T790M and minor mutations, MET amplification and activation of MET/HGF axis, acquiring an epithelial to mesenchymal transition (EMT) signature, and transformation from NSCLC into small cell lung cancer (SCLC) (6-11). More recently, AXL kinase activation and loss of the EGFR-mutant allele have been reported as possible mechanisms of resistance, but it is likely that additional mechanisms remain to be identified (12, 13).

Establishment of resistant sublines is a common experimental method to investigate the mechanism of drug resistance. The properties of resistant cells can vary according to the experimental methods used in the developing process. For example,
endometrial cancer, cisplatin-resistant cells established by stepwise escalation exposure and high-concentration exposure methods showed different cellular properties that may be either a cause or result of drug resistance (14). This fact suggests that the mechanism of EGFR-TKI resistance may vary according to in vitro culture conditions, resulting in finding of novel features of resistant cells. While the majority of previously reported cells that were resistant to EGFR-TKI were established with stepwise escalation of EGFR-TKI concentration, we successfully established resistant cells with the high-concentration exposure method as well as the stepwise escalation method, and identified novel features of cells resistant to EGFR-TKI. The purposes of this study were to investigate the acquired mechanism of resistance to EGFR-TKI and to explore strategies to overcome resistance to EGFR-TKI.

Materials and Methods

Cell lines and reagents

EGFR-mutant HCC827 (exon19del E746–A750), PC-9 (exon19del E746–A750), HCC4006 (exon19del L747–E749), and HCC4011 (L858R) cells were used. These cell lines except for PC-9 were established by one of the authors (AFG). PC-9 was obtained
from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). All the cell lines were
cultured in RPMI 1640 media supplemented with 10 % fetal bovine serum (FBS), and
grown in a humidified incubator with 5 % CO₂ at 37°C. EGFR-TKI resistant sublines
were established by two different methods: parental cells were cultured with stepwise
escalation of concentrations of gefitinib from 5 nM to 2 μM over 6 months (stepwise
escalation method), or initially high concentration of gefitinib (2 μM) over 6 months
(high-concentration method). Finally, gefitinib-resistant sublines named as
HCC827-GR-step, PC-9-GR-step, HCC4006-GR-step, and HCC4011-GR-step were
established by stepwise escalation method, and HCC827-GR-high1, HCC827-GR-high2,
PC-9-GR-high, HCC4006-GR-high, and HCC4011-GR-high were established by
high-concentration method. Regarding HCC827-GR-high1 and high2, these two
sublines were independently established from different cultures with high-concentration
method. The identities of all the parental and resistant cells were confirmed by analyzing
the short tandem repeat profile using the Cell ID System (Promega, Madison, WI) and
ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), according to
the manufacturer’s instructions. Clonal resistant cells were isolated by limiting dilution.
Details about the reagents used in the cell proliferation assay and the antibodies for Western blot analysis, fluorescence immunocytochemistry, and immunohistochemistry are included in Supplementary Methods.

**Determination of cell proliferation**

Cell proliferation was determined by a modified MTS assay with CellTiter® 96 Aqueous One Solution Reagent (Promega) as previously reported (15). The antiproliferative effects are shown as IC\textsubscript{50}, which is the concentration of the drug required to inhibit cell proliferation by 50%.

**Western blot analysis**

The detailed protocol for the Western blotting has been described previously (15). Monoclonal anti-actin antibody, used as an equal loading control, was purchased from Merch KGaA (Darmstadt, Germany). The following secondary antibodies were used: goat anti-rabbit or anti-mouse IgG-conjugated horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA). To detect specific signals, the membranes were examined using ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

**Phospho-receptor tyrosine kinase (RTK) array and phospho-kinase arrays**
A Human Phospho-RTK Array Kit and a Human Phospho-kinase Array Kit (R&D Systems, Minneapolis, MN) were used to measure the relative level of tyrosine phosphorylation of 42 distinct receptor tyrosine kinases (RTKs) and the relative level of phosphorylation of 46 distinct intracellular kinases. Both arrays were performed according to the manufacturer’s instructions.

**Fluorescence immunocytochemistry**

The cells were cultured and fixed by 4% formaldehyde on chamber slides. Primary antibodies against EGFR, E-cadherin, vimentin, ALDH1A1, and ABCB1 were used. Further details are provided in Supplementary Methods.

**DNA and RNA extraction**

Genomic DNAs were isolated from cell lines, frozen tumors, or paraffin embedded tumor by using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), standard phenol-chloroform (1:1) extraction followed by ethanol precipitation, or QIAmpl DNA FFPE Tissue Kit (Qiagen), respectively. Total RNAs were extracted from cell lines using RNeasy Plus Mini Kit (Qiagen). The complementary DNA (cDNA) was synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to the manufacturer’s instructions.
**Direct sequencing, PCR-based length polymorphism assay, and sub-cloning**

We determined the mutational status of *EGFR*, *KRAS*, *NRAS*, and *BRAF* genes by direct sequencing, and PCR conditions are provided in Table S1A. *EGFR* exon19 deletion was also detected with PCR-based length polymorphism assay which have previously reported (16). For sub-cloning, PCR products were cloned into pCR2.1-TOPO vector using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). One hundred clones were randomly selected for PCR-based length polymorphism assay.

**Analyses of copy number by qPCR and FISH assays**

Copy number gains (CNGs) of *EGFR* and *MET* genes were determined by real-time quantitative PCR (qPCR) assay using Power SYBR Green PCR Master Mix (Applied Biosystems) as previously reported (17, 18). Primer sequences are provided in Table S1B. In brief, gene dosage of each target and *LINE-1* gene, a reference gene, was calculated using the standard curve method. Relative copy number of each sample was determined by comparing the ratio of target gene to *LINE-1* in each sample with the ratio of these genes in human genomic DNA (EMD Biosciences, Darmstadt, Germany). Based on our previous study, we defined high-level amplification as values greater than 4 in cell lines and those greater than 5 in clinical samples (17, 18).
A dual-color fluorescence in situ hybridization (FISH) assay was performed using the LSI EGFR spectrumOrange/CEP7 spectrumGreen probe (Vysis, Downers Grove, IL) according to the manufacturer’s instructions. Twenty metaphase spreads and 200 interphase nuclei were analyzed in each slide.

**Hybridoma production and TKI sensitivity analysis**

The parental HCC827 cells were fused with HCC827-GR-high2 using Sendai virus (Hemagglutinating Virus of Japan) envelope (HVJ-E) GenomONE™-CF (Ishihara Sangyo Kaisha Ltd., Osaka, Japan) according to the manufacturer’s instructions. In brief, HCC827 cells stained with PKH26 Red fluorescent Cell Linker Kit (Sigma-Aldrich, St Louis, MO) were mixed at a ratio of 1:1 HCC827-GR-high2 cells stained with PKH67 Green fluorescent Cell Linker Kit (Sigma-Aldrich). The fused cells were confirmed as double-fluorescent positive cells in fluorescent microscopy. Cells were treated with 2 µM of gefitinib and the presence of double-fluorescent positive and single-fluorescent positive cells (HCC827 and HCC827-GR-high2) was examined 14 days after.

**Expression profiling analysis**

RNA from cells was profiled on Illumina HumanHT-12 V4 Expression BeadChip arrays according to the Illumina protocol. The array measures expression levels for over
47,000 transcripts derived from the NCBI RefSeq Release 38. BRB array tools (version 4.2) were used to perform robust spline normalization on background corrected data to generate log2 transformed normalized data. Fold change in expression for individual probes was calculated and probes with fold changes exceeding 2-fold or below 2-fold were considered over- and under-expressed, respectively (Table S2).

**mRNA and miRNA expression analysis by qRT-PCR**

Messenger RNA (mRNA) expression analysis by quantitative real-time RT-PCR (qRT-PCR) was performed on cDNA using TaqMan probes and the TaqMan Universal PCR Master Mix (Applied Biosystems). In miRNA expression analysis, the miRNA was isolated with TaqMan MicroRNA Cells-to-CT Kit (Ambion, Austin, TX), and RT reaction was performed with TaqMan MicroRNA Reverse Transcriptional Kit systems (Applied Biosystems) using TaqMan single RT primers for each miRNA. Primer and probe sets (Table S1C, D) were purchased from Applied Biosystems and used according to manufacturer’s instructions. PCR amplification was conducted on an ABI StepOne Real-Time PCR Instrument (Applied Biosystems) and gene expression was calculated using the comparative CT method. Three replicates per sample were assayed for each gene. To quantify the relative changes in gene expression, the $2^{-\Delta\Delta C_T}$ method was used and reactions were normalized to endogenous control gene *glyceraldehyde-3-phosphate*.
dehydrogenase (GAPDH) expression levels in mRNA expression analysis, and miR-374 expression level in miRNA expression analysis, respectively.

**DNA methylation analysis**

DNA was subjected to bisulfate treatment using Epitect Bisulfite Kit (Qiagen) according to the manufacturer’s protocol. DNA methylation status was examined by bisulfite genomic sequencing and MSP as previously reported (19). Primers are listed in Table S1E, F.

**Sphere formation assays in serum-free cultures**

A total of 5 × 10^3 of cells were plated in 24-well plates with Ultra-Low Attachment surface (Corning Inc., Lowell, MA), and cultured in serum-free DMEM/F12 (Invitrogen) supplemented with 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL basic fibroblast growth factor (bFGF) (all from Sigma-Aldrich). The numbers of spheres exceeding 150 µm in size for each well were counted by microscope after 14 days of culturing.

**Side-population analysis**

The basic protocol for side-population (SP) analysis was based on Goodell et al (20). Cells were resuspended at 1 × 10^6/mL in pre-warmed RPMI 1640, and Hoechst
33342 dye (Sigma-Aldrich) was added to a final concentration of 5 µg/mL in the presence or absence of verapamil (30 µg/mL; Sigma-Aldrich). The cells were then incubated at 37°C for 75 min with intermittent shaking. Propidium iodide (Sigma-Aldrich) was added to the cells, to a final concentration of 1 µg/mL, to gate the viable cells. The cell preparations were filtered through a 40 µm cell strainer to obtain single cell suspension. The cell samples were then analyzed on a BD FACS Vantage SE (BD Biosciences, Franklin Lakes, NJ) cell sorter. The SP was identified as a group of cells that did not take up the Hoechst dye, a characteristic abolished with verapamil treatment (20). These analyses were performed at ReproCELL (Yokohama, Japan).

**Tumor cell implantation experiments**

HCC827, HCC827-GR-high1, and HCC827-GR-high2 cells were subcutaneously injected into the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice purchased from Charles River (Yokohama, Japan). Groups of mice were inoculated with each cell line at 5 x 10^6. Tumor growth was monitored and individual tumor volumes were measured using a digital caliper and approximated according to the formula \( V = \frac{1}{2}ab^2 \) (a: being the long diameter and b: the short diameter of the tumor). At the end of experiments, mice were sacrificed after 4 weeks and tumors were harvested, measured, photographed, and pathologically examined.
Immunohistochemical analyses of clinical samples

Tumor samples were obtained at Okayama University Hospital with patients’ consent under Institutional Review Board–approved protocols. Biopsied samples after acquisition of EGFR-TKI resistance were fixed in 10% formaldehyde and embedded in paraffin. IHC staining with ALDH1A1 (diluted 1:400 in PBS), ABCB1 (1:200), E-cadherin (1:1000), and Vimentin (1:200) was performed. The detailed protocol for the IHC staining has been described previously (21).

Statistical analyses

All data were analyzed using JMP v 9.0.0 software (SAS Institute Inc., Cary, NC). P < 0.05 was considered significant. All tests were two-sided.

Results

Genotypic mechanisms of acquired resistance to EGFR-TKIs

Four cell lines (HCC827, PC-9, HCC4006, and HCC4011) with TKI-sensitive EGFR mutations were exposed to gefitinib by 2 different methods: stepwise escalation (GR-step series) and high-concentration exposure (GR-high series). From these efforts, 9 sublines resistant to gefitinib were established: HCC827-GR-step, PC-9-GR-step,
HCC4006-GR-step, HCC4011-GR-step, HCC827-GR-high1, HCC827-GR-high2, PC-9-GR-high, HCC4006-GR-high, and HCC4011-GR-high. The IC$_{50}$ values against gefitinib and erlotinib of these 9 resistant sublines exceeded 5 µM (Table 1). The expressions of EGFR and its effector proteins in these cell lines were profiled (Fig. S1A).

To explore the genotypic changes following acquired resistance to EGFR-TKIs, we examined the genomic DNA of both parental and resistant cells. In sublines established with the stepwise escalation method, qPCR revealed that HCC827-GR-step and HCC4011-GR-step exhibited $MET$ amplification (Fig. S1B), while PC-9-GR-step showed the presence of $EGFR$ T790M mutation detected by direct sequencing (Fig. S1C). Among sublines established with the high-concentration method, only HCC4011-GR-high showed $MET$ amplification (Fig S1B). These resistance mechanisms were consistent with the results of previous studies (22-24). Neither T790M nor $MET$ amplification was observed in any of the other 5 sublines (HCC4006-GR-step, HCC827-GR-high1, HCC827-GR-high2, PC-9-GR-high, and HCC4006-GR-high). No resistant sublines harbored secondary mutations in the $KRAS$, $NRAS$, or $BRAF$ gene.

After acquiring resistance to EGFR-TKIs, all sublines except HCC827-GR-high2 retained $EGFR$ mutations, copy number gain, and protein expression, as determined by direct sequencing, qPCR, and Western blotting, respectively (Fig. S1). Originally,
Parental HCC827 cells showed focal amplification of EGFR with 32.3 copies estimated by FISH and qPCR assays. In HCC827-GR-high2, qPCR, PCR-based length polymorphism and FISH assays revealed progressive decrease in the EGFR mutant allele through the course of passages leading to the disappearance of focally amplified EGFR alleles (Fig. 1). Sub-cloning of the PCR product from HCC827-GR-high2 produced 100 of 100 clones exhibiting wild-type EGFR instead of the exon19 deletion, as determined by PCR-based length polymorphism assay. In addition, 3 sublines from independent single cells were established from HCC827-GR-high2. No mutant alleles were detected in 2 of these sublines, while a small population of mutant alleles was identified in the third subline as determined by direct sequencing. These data indicate that the focal amplification of the EGFR gene, which was considered a mutant allele, disappeared in HCC827-GR-high2.

**Phenotypic change in acquiring resistance**

Microscopically, each of the 4 sublines, HCC827-GR-high1, HCC827-GR-high2, HCC4006-GR-high, and HCC4006-GR-step, exhibited a spindle cell-like morphology that was different from their parental cell lines. As expected, these 4 sublines displayed EMT features in Western blotting and fluorescence immunocytochemistry for E-cadherin.
and vimentin (Fig. S2A, B). In contrast, PC-9 and HCC4011 cells and the derived resistant sublines did not show EMT features.

Expression profile of mRNA and kinases in HCC827 cells

Because we had a great interest in HCC827-GR-high2 whose amplified EGFR-mutant alleles disappeared, we examined the mRNA expression profile in HCC827-GR-high2 by using cDNA microarray. Details of this expression profile are presented in Table S2. We identified up-regulation of many genes, and particularly noticed up-regulation in the expression of genes encoding aldehyde dehydrogenase isoform 1 (ALDH1A1) and ABC transporters, which were observed in the cells with stem cell-like properties. The up-regulation of stem cell-related markers such as ALDH1A1, ABC transporters, and CD44 was confirmed by qRT-PCR (Fig. 2). In addition, the up-regulation of ALDH1A1 in HCC827-GR-highs was confirmed by Western blotting and fluorescent immunocytochemistry (Fig. S2C, D). ALDH1A1 was not detected in the other sublines.

We performed phospho-receptor tyrosine kinase array and phospho-kinase array to find no significant differences including AXL between parental HCC827 and HCC827-GR-highs, except for the down-regulation of phosphorylated EGFR protein in HCC827-GR-highs (Fig. S3).
Expression status of miR-200 family in resistant cell lines with EMT features

The result of cDNA microarray in HCC827-GR-high2 indicated the up-regulation of Zinc-finger enhancer binding (ZEB) transcription factors (ZEB1 and ZEB2), which are crucial EMT activators (25-27). Because EMT was reported to be regulated by miR-200 family targeting ZEB1 and ZEB2, we analyzed the expression status of the miR-200 family by qRT-PCR. As shown in Fig. 3A, miR-200a, miR-200b, and miR-200c were down-regulated in both HCC827- and HCC4006-derived resistant sublines with EMT features. In particular, miR-200c was extensively down-regulated in HCC827-GR-highs compared to parental HCC827. To investigate the mechanism of down-regulation of miR-200 family, we examined the methylation status of miR-200 family by methylation-specific PCR (MSP) (Fig. 3B) and bisulfite genomic sequencing (data not shown). Our results indicate that miR-200 families were methylated and their expressions were recovered following treatment with 5-aza-2'-deoxycytidine (Fig. 3C).

Stem cell-like property in acquiring resistance

Up-regulation of ALDH1A1 and ABC transporters suggests that HCC827-GR-highs may acquire stem cell-like properties. Thus, we quantified the SP by dual wavelength flow cytometry. We found an extreme increase in the number of SP cells in HCC827-GR-highs as illustrated in Fig. 4A. The majority of the SP fraction in
HCC827-GR-highs was eliminated in the presence of verapamil, indicating the specificity of the SP. In addition, we performed a sphere formation assay to examine the cellular functional features of stem cell-like properties. We found that HCC827-GR-highs acquired higher ability to form spheres in suspension culture compared to parental HCC827 and HCC827-GR-step (Fig. 4B). To examine the stem cell-like properties in vivo, we performed tumor transplantation experiments (28, 29). We subcutaneously implanted HCC827, HCC827-GR-high1, and HCC827-GR-high2 cells to examine the tumor-forming capability in NOD/SCID mice. We found that HCC827-GR-highs exhibited higher tumorigenicity than parental HCC827 cells did. The HCC827-GR-highs established larger tumors with shorter latencies than the parental HCC827 cells did (Fig. 4C). HCC827-GR-highs showed a significant increase in the expression of ALDH1A1, as indicated by immunohistochemical (IHC) staining (Fig. S4). These results further indicate that HCC827-GR-highs acquired stem cell-like properties. Of interest, the histological finding of HCC827-GR-highs was different from that of parental HCC827 (Fig. S4). Even after culturing of HCC827-GR-highs with gefitinib-free medium for 6 months, HCC827-GR-high2 still exhibited both the EMT and stem cell-like features, and did not exhibit production of EGFR mutant-specific protein or further increase of either mutant allele or EGFR copy number (Fig. S5).
appearance of stem cell-like properties in HCC827 resistant sublines established by high-concentration method was independently confirmed in 3 additional different culture dishes.

**Gefitinib sensitivity of HCC827 and HCC827-GR-high2 hybridoma**

A hybridoma model was generated with parental and resistant cells to distinguish whether the causative factor for EGFR-TKI resistance in HCC827-GR-high2 that exhibited loss of mutant EGFR alleles, EMT, and stem cell-like properties involved either loss or gain of function. A hybridoma cell line was generated by fusing HCC827 with HCC827-GR-high2, which was then treated with gefitinib. The hybridoma exhibited resistance to gefitinib at a concentration of 2 µM, indicating that the resistant cells had acquired drug-resistant properties to parental cells, rather than having lost drug sensitivity.

**Sensitivity to various drugs in each cell line**

Drug sensitivities against various agents are shown in Table 2. The sublines of HCC827, PC-9, and HCC4006 generated under high concentrations of gefitinib exhibited higher IC$_{50}$ values for docetaxel and paclitaxel than those of parental cells. Moreover, we examined the antitumor effects of HDAC inhibitors (trichostatin A [TSA] and vorinostat
[SAHA]) and a proteasome inhibitor (bortezomib). These inhibitors produced moderate to strong antitumor effects in both parental and resistant cells, which did not acquire resistance to these drugs (Table 2) (30, 31).

**Molecular profile of EGFR-TKI resistant clinical samples**

Clinical tumor samples, which showed acquired resistance to TKIs, were collected both before and after TKI treatment and examined genetically and immunohistologically (Table 3). Among 16 cases with acquired resistance to TKIs, corresponding pre-treatment samples were available in 3 cases (no. 1, 5, and 16). After acquiring resistance, 3 cases exhibited *EGFR* T790M mutation, and 2 exhibited *MET* amplification. In addition, some samples harboring EMT features (based on E-cadherin and vimentin expression status) also exhibited expression of stem cell-related markers ALDH1A1 and ABCB1, determined by IHC staining (Fig. 4D). In total, 5 out of 16 cases exhibited ALDH1A1 expression in samples that showed acquired resistance to TKI. In 3 cases whose pre-treatment samples were available, ALDH1A1 was not expressed prior to TKI treatment. Among them, 2 cases (no. 5 and 16) exhibited ALDH1A1 expression after acquiring resistance to TKI. Of note, case no.5 was a patient with recurrent lung cancer after surgery and no other anti-cancer drugs except gefitinib and erlotinib were administrated.
Discussion

In this study, we demonstrated that the method of drug exposure in cell culture influences the mechanisms of acquired resistance to EGFR-TKI. Studies using EGFR-mutant cell lines have indicated that the different types of resistance mechanisms are determined within the individual cell lines in response to certain conditions. For instance, both PC-9 and H3255 are known to develop resistance to EGFR-TKIs through $EGFR$ T790M mutation as a result of stepwise escalation exposure (22, 23). On the other hand, HCC827 is known to overcome EGFR-TKI through $MET$ amplification (8, 32). Interestingly, Suda et al. reported that HCC827 cells developed the T790M mutation under the inhibition of MET signaling, suggesting that the microenvironment of the tumor cells influenced the mechanisms of resistance (32). Indeed, in an analysis using multiple gefitinib refractory tumors obtained from autopsies, the lesions from patients exhibited T790M and/or $MET$ amplification depending on the lesion sites (32). In addition to these resistance mechanisms accompanying somatic gene alterations, recent studies have indicated that some biological signatures such as acquisition of EMT phenotype and transformation to SCLC were associated with EGFR-TKI resistance, the causative genetic alterations of which are not known (11, 33, 34). EMT was observed in
HCC4006 sublines and HCC827-GR-highs, but not in HCC827-GR-step sublines. Summarily, these observations suggest that cancer cells might be able to enact different mechanisms based on the microenvironment to survive and escape cell death from apoptotic pressure of EGFR-TKI.

Among identified EMT-related genes, ZEB1 expression was reported to be most significantly correlated with mesenchymal phenotype in human malignancies including NSCLC (25, 26). The cDNA array revealed up-regulation of ZEB1 and ZEB2 expression in HCC827-GR-high2 and we consequently focused on the miR-200 family, which is reported to repress ZEB1 as a key regulator of EMT (35). Indeed, our results indicate down-regulated expression of the miR-200 family, especially miR-200c, in resistant sublines with EMT features. Moreover, a recent study has indicated that loss of miR-200c expression with DNA methylation is associated with chemoresistant phenotype in NSCLC (36). Of note, it is now known that EMT activators not only activate cellular motility but are also associated with the maintenance of stem cell properties and cell survival (37, 38). Our findings present the novel insight that the repression of miR-200 family by DNA methylation is responsible for EMT during the acquisition of resistance to EGFR-TKI.
As an important finding, some resistant cells with EMT signatures exhibited stem cell-like properties. Cancer-stem cells (CSCs), which are characterized by the capacity for pluripotency and self-renewal, have been attracting interest as a source of cancer cells (39). The significance of stem cell-like properties in lung cancer has been investigated in both basic and clinical research (40, 41). However, the CSC of lung cancers remains a subject of ongoing research, and its specific makers have not yet been identified. In our study, several cell surface proteins, including CD133, that may be candidate markers of general CSC, were not up-regulated in resistant sublines. These results collectively suggest that understanding and overcoming drug resistance from the viewpoint of stem cell identity may present new and challenging opportunities.

We examined the sensitivity of various types of drugs, including conventional chemotherapeutic agents, HDAC inhibitors, and proteasome inhibitor, to resistant sublines. HCC827-GR-highs, HCC4006-GR-high, and PC-9-GR-high exhibited significant resistance to docetaxel and paclitaxel, but not to cisplatin. This result is reasonable because docetaxel and paclitaxel, unlike cisplatin, are considered to be effluxed by the ABC transporter system, which was up-regulated in these sublines. These results would lead to selection of appropriate chemotherapeutic drugs following acquired resistance to EGFR-TKI.
In our study, clinically usable HDAC inhibitor and proteasome inhibitor SAHA and bortezomib exhibited similar antitumor efficacy for both parental and resistant cells. Clinical studies have demonstrated that SAHA is effective for NSCLC when combined with other cancer therapies (42, 43). Notably, HDAC inhibitors are reported to be effective in treating chronic myelogenous leukemia, in which stem cells appeared after the acquisition of resistance to imatinib mesylate (44). The proteasome inhibitor, bortezomib failed to produce antitumor effects either alone or in conjunction with other drugs in a clinical trial, although preliminary data suggested that bortezomib as a single agent may show antitumor activity in NSCLC patients (45, 46). However, current research is rapidly working toward the development of next-generation proteasome inhibitors (47), and our data indicating that bortezomib activity was not influenced by the EGFR-TKI resistance state may be useful for the future development of proteasome inhibitors.

Our results support the usefulness of *in vitro* experiment to investigate the mechanisms of resistance to EGFR-TKI. In the pharmacokinetic analysis of gefitinib, the plasma concentration of gefitinib was found to reach a steady-state level within 7 days, in most of the cases (48). Nakamura et al. reported that the plasma concentration of gefitinib in patients receiving 250 mg gefitinib daily ranged from 115 to 2012 ng/mL (0.257 to...
4.502 µM) on day 3 (D3) and 126 to 2926 ng/mL (0.282 to 6.547 µM) on day 8 (D8), while the median D8-to-D3 ratio was 1.578 and ranged from 0.758 to 6.094 (49). These results indicate considerable variation in plasma concentration and the pharmacokinetics of gefitinib according to individual patients. Of note, D8-to-D3 ratio under 1.0 indicates that drug concentration may reach a plateau shortly after initiation of gefitinib treatment. This condition may be similar to that of drug contact to cells in our high-concentration method. In addition, Haura et al. reported that tumor levels during treatment with gefitinib were higher than, but not related to, plasma levels (50). Further resistance-related research will require additional elaboration regarding culture conditions and microenvironment.

It is still not known what caused the emergence of stem cell-like properties in HCC827-GR-highs; however, 2 possibilities should be considered: (i) high-concentration exposure induces cells with stem cell-like properties; and (ii) the cells with stem cell-like properties existed as minor clones in HCC827 prior to gefitinib exposure, and these were finally selected as the major clones. For the latter possibility, it is also not clear as to why cells with stem-like properties appeared as the major population only from the high-concentration exposure method.
In conclusion, culture conditions with EGFR-TKI appear to influence the mechanism of acquired resistance, suggesting that the microenvironment is a determinating factor for the mechanisms underlying acquisition of resistance to EGFR-TKI. Our study indicated that EGFR-TKI treatment induced stem cell-like properties and is associated with EGFR-TKI resistance.

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43. Traynor AM, Dubey S, Eickhoff JC, Kolesar JM, Schell K, Huie MS, et al.


Figure legends

Fig. 1. Loss of EGFR mutant allele and progressive decrease of EGFR copy number in HCC827-derived resistant subline. HCC827-GR-high2 showed progressive decrease in the EGFR mutant allele through the course of passages leading to the disappearance of focally amplified EGFR alleles. (A) Fluorescence immunocytochemistry of EGFR E746-A750 deletion mutation-specific protein (green; mutation-specific protein, blue; nucleus), (B) FISH (red; EGFR, green; CEP7, blue; nucleus), (C) progressive decrease in the EGFR mutant allele by qPCR and PCR-based length polymorphism assay. P-: passage. Scale bars: (A) 100 µm, (B) 10 µm.

Fig. 2. Relative gene expression levels of stem cell-related markers by quantitative RT-PCR. The mRNA expressions of ALDH1A1, ABCB1, ABCG2, and CD44 were confirmed by quantitative real-time RT-PCR. The expression of stem cell-related markers ALDH1A1, ABCG2, and CD44 were higher than that of the parental line in HCC827-GR-highs. In addition, the expression of ABCB1 was significantly higher than that of the parental line in HCC827-GR-step, HCC827-GR-highs, PC-9-GR-high, and HCC4006-GR-high.

Fig. 3. CpG island hypermethylation-associated silencing of the miR-200 family in acquired resistance to EGFR-TKI sublines with EMT features. (A) The expression
of miR-200 family was extensively down-regulated in resistant sublines with EMT features compared to parental lines by quantitative RT-PCR. (B) The methylation specific PCR (MSP) revealed the hypermethylation of miR-200 family in resistant cells with EMT features. (C) The miR-200 family expressions were recovered following treatment with DNA-demethylating agent 5-aza-2′-deoxycytidine (5-Aza) in miR-200 family methylated cells. M: methylated; U: unmethylated

Fig. 4. Emergence of the stem-cell like properties in acquiring resistance to EGFR-TKI cells. (A) Side population (SP) analysis shows the extreme increase of SP cells in HCC827-GR-highs compared to parental cells. The majority of the SP fraction in HCC827-GR-highs was eliminated in the presence of verapamil. (B) By sphere formation assay, HCC827-GR-highs acquired high ability to form spheres in suspension culture. (C) The HCC827-GR-highs established larger tumors with shorter latencies than the parental HCC827 cells did in NOD/SCID mice, indicating HCC827-GR-highs exhibited higher tumorigenicity than parental HCC827 cells. (D) Representative images of IHC staining of pretreatment and recurrent tumor after EGFR-TKI treatment (case no. 5). The post-resistant biopsy (right panels) demonstrates an EMT feature with positive IHC staining for vimentin with expression of stem cell-related marker ALDH1A1. Scale bars: 100 μm.
<table>
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<tr>
<th>Cell Line</th>
<th>Gefitinib exposure</th>
<th>Gefitinib IC$_{50}$ (µM)</th>
<th>Erlotinib IC$_{50}$ (µM)</th>
<th>EGFR T790M</th>
<th>MET amp</th>
<th>Other</th>
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<td>&gt; 10</td>
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<td>-</td>
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<tr>
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<td>No</td>
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<tr>
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<td>&gt; 10</td>
<td>Yes</td>
<td>No</td>
<td>-</td>
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<td>&gt; 10</td>
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<td>EMT</td>
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<td>No</td>
<td>EMT</td>
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<td>HCC4011</td>
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<td>6.48</td>
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<td>-</td>
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</table>

N/A: not applicable; amp: amplification; EMT: epithelial to mesenchymal transition; WT: wild type.
Table 2. IC<sub>50</sub> values (µM) against various agents.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Chemotherapeutic agent</th>
<th>HDAC inhibitor</th>
<th>Proteasome inhibitor</th>
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<tr>
<td></td>
<td>CDDP</td>
<td>DOC</td>
<td>PTX</td>
</tr>
<tr>
<td>HCC827</td>
<td>4.51</td>
<td>0.0021</td>
<td>0.0032</td>
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<tr>
<td>HCC827-GR-step</td>
<td>4.29</td>
<td>0.0020</td>
<td>0.0020</td>
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<tr>
<td>HCC827-GR-high1</td>
<td>4.45</td>
<td>0.022*</td>
<td>0.15*</td>
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<td>HCC827-GR-high2</td>
<td>8.04</td>
<td>0.51*</td>
<td>1.46*</td>
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<td>PC-9</td>
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<td>0.824*</td>
<td>1.10*</td>
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</table>

CDDP: cisplatin; DOC: docetaxel; PTX: paclitaxel; TSA: trichostatin A; SAHA: vorinostat.

*The ratio of the IC<sub>50</sub> value in each resistant line to the parental line is higher than 10 times.
Table 3. Acquired resistance to EGFR-TKIs; clinical samples.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>EGFR mutation</th>
<th>Genetic alteration</th>
<th>Immunohistochemistry</th>
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<td>Vimentin</td>
</tr>
<tr>
<td>1</td>
<td>88</td>
<td>F</td>
<td>L858R</td>
<td>T790M</td>
<td>- / -</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>F</td>
<td>19 del</td>
<td>T790M</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>53</td>
<td>F</td>
<td>19 del</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>F</td>
<td>19 del</td>
<td>Loss of EGFR-mut</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>F</td>
<td>L858R</td>
<td></td>
<td>- / +</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>M</td>
<td>19 del</td>
<td></td>
<td>-</td>
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<tr>
<td>7</td>
<td>73</td>
<td>F</td>
<td>19 del</td>
<td>MET amp</td>
<td>-</td>
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<td>8</td>
<td>62</td>
<td>F</td>
<td>19 del</td>
<td></td>
<td>+</td>
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<td>9</td>
<td>70</td>
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<td>19 del</td>
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<td>-</td>
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<tr>
<td>16</td>
<td>71</td>
<td>F</td>
<td>L858R</td>
<td></td>
<td>- / +</td>
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</tbody>
</table>

Among 16 cases with acquired resistance to TKIs, corresponding pre-treatment samples were available in 3 cases (no. 1, 5, and 16). Del: deletion; mut: mutation; amp: amplification; N/A: not applicable.
Figure 1

A

HCC827-parental

HCC827-GR-high2 (P-123)

B

HCC827-parental

HCC827-GR-high2 (P-123)

C

<table>
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<tr>
<th>EGFR copy number (qPCR)</th>
<th>Parental</th>
<th>P-10</th>
<th>P-22</th>
<th>P-46</th>
<th>P-60</th>
<th>P-108</th>
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<td>Mutant (123 bp)</td>
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Figure 2

### ALDH1A1

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<td>parent</td>
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<tr>
<td>GR-step</td>
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<td>GR-high1</td>
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### ABCB1

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<td>GR-step</td>
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<tr>
<td>GR-high1</td>
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<td>GR-high2</td>
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### ABCG2

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<tr>
<td>GR-step</td>
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<tr>
<td>GR-high1</td>
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### CD44

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<tr>
<td>GR-step</td>
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<tr>
<td>GR-high1</td>
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<tr>
<td>GR-high2</td>
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<td></td>
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</table>
Figure 3

A

miR-200a

miR-200b

miR-200c

Relative expression

0.2 0.4 0.6 0.8 1.0 1.2 1.4

parental GR-step GR-high1 GR-high2 parental GR-step GR-high1 GR-high2 parental GR-step GR-high1 GR-high2

HCC827 HCC4006 HCC827 HCC4006 HCC827 HCC4006

B

HCC827 PC-9 HCC4006 HCC4011

miR-200ba429

M

U

miR-200c141

M

U

C

Relative expression

0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8

parental GR-step GR-high1 GR-high2 parental GR-step GR-high1 GR-high2 parental GR-step GR-high1 GR-high2

HCC827 HCC827-GR-step HCC827-GR-high1 HCC827-GR-high2

Experimental details are provided in Supplementary Methods.
Figure 4

A

HCC827 + Verapamil

HCC827-GR-high1 + Verapamil

HCC827-GR-high2 + Verapamil

B

HCC827 HCC827-GR-high2

Sphere number

Tumor volume (mm²)

C

D

Primary lung tumor Recurrent tumor

Vimentin

ALDH1A1
Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells.

Kazuhiko Shien, Shinichi Toyooka, Hiromasa Yamamoto, et al.

Cancer Res Published OnlineFirst March 29, 2013.

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